FERMENTATION OF SULFITE SPENT LIQUOR

by

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Abstract

Fermentation of sulfite spent liquor with Propionibacterium freudenreichii was done to produce volatile acids (acetic and propionic) and Vitamin $B_{12}$. It was found that in addition to producing these compounds some reduction in the pollution potential (COD) of this waste product was achieved.

Better growth resulted if the spent liquor was first treated to remove lignin and calcium compounds.

An existing spectrophotometric assay technique for measuring Vitamin $B_{12}$ content was modified for use in the presence of sulfite spent liquor.

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## 1. Introduction

The discharge of waste liquor from industrial operations can lead to a major water pollution problem. This is particularly true of the effluent discharged from sulfite pulping operations. The pollution caused by this sulfite spent liquor (SSL) results from suspended solids, dissolved organic substances, and chemicals which are toxic to the inhabitants of the receiving water and perhaps even to the users of this water (1-8).

In the sulfite process about 8 tons of spent liquor are discharged per ton of pulp produced. In this amount of spent liquor are found 1000 lbs of lignin complexes, 500 lbs of wood sugars (reducing substances) and about 20 lbs of suspended solids (9).

Suspended solids may be removed by screening, filtration, settling, or flotation (1). This suspended matter, which is mostly fibre or bark, causes irritation of the gill tissue of fish. Also as cellulose is relatively resistant to decay, this fibre debris tends to blanket the bottom of the receiving stream creating anaerobic conditions on the bottom. This smothers the life at the stream bottom and may produce unpleasant smelling gases. Where regulations are in force a stipulated maximum is 0.3 lbs of suspended solid per U.S. gallon discharged (10). The average industry discharge is considerably higher.

Toxic materials such as resin acid soaps, mercaptans, etc. are usually neutralized by pH adjustment. However not much is really known about this.

Lignin complexes and tannins cause water discoloration as well as foaming when discharged from the mill. Such conditions are, if nothing

else, unsightly. Foaming tends to reduce the efficiency of most solids
removal techniques.

Dissolved, oxidizable chemicals such as wood sugars, lignin
compounds, etc. are naturally oxidized by microbes in the water. This
process removes oxygen from the receiving stream; 400 lbs or more of
oxygen may be required to oxidize the effluent discharged in the pro-
duction of 1 ton of sulfite pulp. Since water saturated with oxygen
contains only 8-11 ppm of oxygen, oxygen depletion occurs in the water
rather rapidly unless oxygen can be dissolved in the water at a rate
equal to or greater than the rate at which it is being used up. If
this cannot be done the aquatic fauna suffocate from lack of oxygen.

The solution to these pollution problems may be in finding
a use for the spent liquor, or a treatment which effectively neutralizes
it. It is possible that the sulfite process will be displaced by the
kraft process and thus the problem will no longer exist, as the water
pollution load from a kraft mill is much less than that of a sulfite mill.
However, the sulfite process is still with us and it may well be that
the kraft process will be legislated out of existence unless it can
solve its air pollution problems. Thus there is some justification
for studying the utilization of sulfite spent liquors. Some uses for
the liquor have been proposed (11-19); these are in road binding, adhe-
sives, and as a fuel. These are low cost products and require concentra-
tion of the liquor via a costly evaporation process. Small quantities
of lignin in spent liquor are used in the manufacture of vanillin (20,
21, 22), however presently operating plants have sufficient capacity
to meet world demands. Some of the wood sugar in the sulfite spent

liquor is pentose from which furfural can be manufactured by acid distillation (23) or by an ultrasonic technique (24) but neither process has proven attractive economically. Fermentation of the wood sugars has been suggested. A number of mills are in existence producing ethanol and yeast by fermentation of the sugars in the sulfite spent liquor (25, 26, 27). Since considerable amounts of oxygen are required to oxidize these sugars, their removal might effectively lower the pollution load of the effluent. However it should be noted that in Canada, U.S.A., U.S.S.R. and Japan little recovery in this form is practiced.

Let's look at fermentation in more detail. Stream pollution must be reduced. This requirement will result from legislation, or enlightened management attitudes. If it must be reduced any return on the investment required to reduce it will be desirable. The more valuable the product from a spent liquor treatment is, the greater is the chance for producing an acceptable return on investment. Jensen (28) and Van Niel (29) suggested the use of propionibacteria for the production of organic acids by fermentation of wastes containing sugars. A valuable byproduct from such a fermentation would be vitamin $B_{12}$ (VB$_{12}$) (30, 31, 32, 33).

The production of acetic acid and propionic acid from sulfite spent liquor (Ca base) by fermentation with propionibacteria has been reported (34, 35, 36, 37). It is known that some species of propionibacteria produce VB$_{12}$ if cobalt ion is present in low concentration (38, 39, 40, 41). Some microorganisms make VB$_{12}$-like compounds which have little or no biological activity but it has been shown that

Propionibacterium freudenreichii produces active 5,6-dimethyl

benzimidazole hydroxo cobalamin (OHB$_{12}$) (42) inside its cell body

(43). Some cobamides found in microorganisms are shown in Table 1.

The VB$_{12}$ yields recorded by previous workers from the fermentation of

materials other than pulp mill wastes are presented in Table 2. The

present work considers the production of VB$_{12}$ and organic acids from

calcium base sulfite spent liquor by fermentation with Propionibacterium

freudenreichii (ATCC 6207).

1-1 Metabolism

Propionibacteria can use both hexose and pentose for growth.

The metabolic pathway for the formation of organic acids has been studied

by many workers (44-54). However the complete system of reactions which

occur inside the cell is not yet fully understood.

According to Phares and Delwiche (50) Propionibacterium shermanii

was able to effect a quantitative conversion of succinic acid to propionic

acid and CO$_2$ under anaerobic conditions. Smith (51), Swick (52), Flavin,

et al (45-49), and Staatman, et al (55) believe that methyl malonyl

CoA, succinyl CoA, and propionyl CoA are intermediates in propionic acid

metabolism. Methyl malonyl CoA is derived from pyruvic acid which is

the end product of sugar metabolism via glycolysis. A suggested meta-

bolic route to propionic acid and acetic acid is shown in Fig. 1.

DBCC (5,6-dimethyl benzimidazolyl cobamide CoA) which is a

co-factor in transmethylation reactions (Fig. 1) is derived from VB$_{12}$

(43, 56, 57, 58). However details of the conversion of VB$_{12}$ to DBCC

are not yet known.

Figure 1

ACIDS METABOLISM

Table 1

Cobamides

| Cobamide | Sources | Reference | Activity (111, 113)[*] | |
| --- | --- | --- | --- | --- |
| | | | L. leichmanii | Ochromonas |
| Hydroxo Cobalamin | P. freudenreichii<br>Streptmyces species | Perlman (42)<br>" | | |
| Pseudo $VB_{12}$<br>(Adenine hydroxo<br>cobalamin) | P. arabinose<br>P. pentosaceum<br>P. shermanii<br>Anaerobes | Perlman (42)<br>"<br>Hodgkin (59)<br>Pfiffner (96) | 40 - 75 | 0.03 |
| Factor A<br>(2-Methyl-$\alpha$ adenyl<br>cobamide) | P. freudenreichii<br>P. thoenii<br>P. pentosaceum | Porter (97)<br>"<br>" | 17 - 40 | slight |
| Factor B<br>(cobinamide) | Calf rumen<br>Nocardia rugosa<br>P. shermanii | Ford (98)<br>Di Marco (99)<br>Pawelkiewitz (100) | 0 | 0.03 |
| Factor C<br>($\alpha$-guanyl cobamide) | N. rugosa<br>E. coli<br>P. shermanii | Barchielli (101)<br>Ford (102)<br>Pawelkiewitz (100) | 10? | 0 |

* Ochromonas and L. leichmanii indicate the microbiological method used to measure the $VB_{12}$ activity.

* Activity is 100 for cyanocobalamin.

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1-2   Vitamin $B_{12}$

$VB_{12}$ was first isolated from liver concentrate (40). Rickes

et al (41) described a microbial synthesis using streptmyces griseus.

Later many other microorganisms were discovered that could synthesize

$VB_{12}$. $VB_{12}$ is essential for normal blood formation and neural function

in mammals as well as playing a role in several other mammallian growth

processes.

$VB_{12}$ is a water soluble vitamin known chemically as a cobalamin.

It is a complex co-ordinating compound containing a tri-valent cobalt

ion with a co-ordination number of six. The complete structural formula

for cyanocobalamin ($CNB_{12}$) which was reported by Hodgkin, et al (59)

is given in Fig. 2. Cyanocobalamin is a neutral molecule; the cyano

group attached to the cobalt atom can be replaced by other ions or

groups to yield other cobalamins such as hydroxocobalamin, chloro-

cobalamin, nitro-cobalamin, or thiocyanocobalamin. Of these hydroxo-

cobalamin is reported to be the most biologically active cobalamin (43).

All these cobalamins can be converted to the stable cyanocobalamin by

treatment with cyanide ion.

$VB_{12}$ is now obtained almost exclusively from microbial sources,

either as a primary fermentation product, or as a byproduct in the

production of certain antibiotics. The importance of microbial pro-

duction of $VB_{12}$ was summarized by Smith (60) as follows:

"It seems probable that the only primary source of $VB_{12}$ in
nature is the metabolic activity of microorganisms; there is no con-
vincing evidence for its elaboration in tissues of higher plants or
animals. It is apparently synthesized by a wide range of bacteria and
actinomycetes though apparently not to any extent by yeast or fungi."

Figure 2  CYANOCOBALAMIN

Highly purified $VB_{12}$ is necessary for the treatment of pernicious anemia and nutritional deficiencies in humans, but at the present time the most promising, large scale market for $VB_{12}$ appears to be as a supplement in animal feeds.

## 1-3 Propionibacterium freudenreichii

This bacteria was found in dairy products and is a gram-positive, non-spore forming, non-motile bacteria. It grows under both aerobic and anaerobic conditions utilizing carbohydrates, polyalcohols, or lactic acid to produce propionic acid, acetic acid, and $CO_2$ (53). Eleven species of propionibacteria have been described (29, 54, 61). On yeast extract-glucose medium P. freudenreichii and P. shermanii grow as small cocci. P. freudenreichii is rod shaped in the synthetic medium (1% Beef extract, 0.3% yeast extract, 1% peptone, 0.5% dextrose, 0.1% soluble starch, 0.05% cysteine hydrochloride, 0.5% sodium chloride, 0.3% sodium acetate and 0.05% agar) but changes its shape to cocci in the sulfite spent liquor medium. A photograph of P. freudenreichii in SSL medium is shown in Fig. 3.

Perlman, et al (42) found that P. freudenreichii produces hydroxocobalamin which is converted to DBCC much faster than cyanocobalamin or other cobalamins (43). Typical production figures for the synthesis of $VB_{12}$ by this bacteria are provided in Table 2.

The optimal temperature for the synthesis of $VB_{12}$ by P. freudenreichii is about 30°C and the optimal pH is 6.5 to 7.0 (62). It has been experimentally demonstrated that a good yield of $VB_{12}$ can not be expected under strictly aerobic or strictly anaerobic conditions (30, 62, 63).

# Figure 3

## P. freudenreichii in SSL media



x 9000

Table   2

VB$_{12}$ Yield of Previous Workers

| Bacterium | Yield (mg/l) | Reference | |
|---|---|---|---|
| P. freudenreichii | 3.0<br>2.4<br>3.0<br>0.4(24hrs)<br>3.7 | Leviton<br>Aso<br>Sudarsky<br>Perlman<br>Riley | (30)<br>(33)<br>(65)<br>(42)<br>(64) |
| P. shermanii | 3.0 | Hinz | (103) |
| Streptmyces  species | 5.7 | Pagona | (104) |
| S. griseus | 0.3 | Wood<br>Dulaney | (110)<br>(105) |
| S. olivaceus | 3.3<br>3.0<br>1.7 | Hall<br>Hester<br>Pfeifer | (106)<br>(107)<br>(63) |
| S. fradiae | 0.7 | Nelson | (108) |
| Bacillus megatherium | 0.45<br>0.48 | Lewis<br>Garibaldi | (109)<br>(62) |
| Aerobacter aerogenes | 0.42 | Hodge | (32) |
| Flavobacterium solare | 0.6 | Petty | (76) |

Riley, et al (64) reported that factor B (VB$_{12}$ less the nucleotide; cobinamide) was formed under anaerobic conditions and that a nucleotide was introduced into factor B under aerobic conditions to give VB$_{12}$. Thus for a good yield of VB$_{12}$ they suggested that the fermentation be conducted in two stages, the first anaerobically to produce factor B and the second aerobically to complete conversion to VB$_{12}$. Aso, et al (33) noted that agitation was necessary for a good yield of product and also that fermentation with agitation but without aeration gave a good yield of VB$_{12}$. Agitation effects on VB$_{12}$ production have also been discussed by Pfeifer (63), Garibaldi (61), and Sudarsky (64).

1-4  Objectives of this research

    The goals of the present work are

    1) to get some quantitative data on the production of VB$_{12}$ and organic acids by fermentation of sulfite spent liquor with P. freudenreichii

    and

    2) to determine if such a fermentation can effectively reduce the pollution load of sulfite spent liquor by removing some or all of the wood sugars and lignins.

2.  Experimental technique

2-1  Analysis of wood sugars

    The total reducing substances in sulfite spent liquor was measured by the method of Sieber (66). This is also Swedish standard method CCA 11 (1941). For further details see appendix 1.

2-2  Volatile acid analysis

    The total volatile acids (propionic acid and acetic acid) in

the fermentation broth were measured by a distillation method and checked by steam distillation. The ratio of propionic acid to acetic acid was measured by steam distillation (67). See appendix 1 for details.

2-3 Lignin analysis

Analysis for lignin in the sulfite spent liquor followed the method of Partansky and Benson (68). Details are given in appendix 1.

2-4 Chemical oxygen demand (C.O.D.)

This was determined by ASTM method D 1252-58T (68). The COD measurement is described in appendix 2.

2-5 Measurement of bacterial growth

Bacterial growth was followed by measuring the turbidity of a suspension of cells produced as follows. A sample of fermentation broth was centrifuged for 15 minutes at 2500 g's to remove the bacteria plus some precipitated solids from the spent liquor. These solids and bacteria were resuspended in distilled water and centrifuged at 100 g's for 1-2 minutes to remove the spent liquor solids. The supernatant was decanted and centrifuged at 2500 g's for 10 minutes to collect the bacterial cells. After washing these separated cells with distilled water, the wash water was removed by a further centrifuging at 2500 g's for 10 minutes. This washing and centrifuging procedure was done twice. Then the cells were dispersed in 10 times the original sample volume of distilled water. For test tube growth tests no dilution was used to measure the turbidity. The turbidity of the suspension was measured at 550 mμ in a Bausch and Lomb spectronic 20 spectrophotometer, and cell suspension concentration

was reported as turbidity.  See appendix 1.

2-6  Vitamin $B_{12}$ analysis

Analysis for $VB_{12}$ may be carried out in various ways (79, 80).
Biological assays (81, 82, 83), microbiological assays (84-90), isotope
analysis (91, 92), paper chromatography (42), or spectrophotometric
techniques (93, 94) may be employed.  Biological assays require facilities
for measuring rat or chick growth, isotope analysis requires specialized
equipment not presently available to us, and paper chromatography analysis
requires calibration with hard to get pure cobamides.  Thus we chose
spectrophotometric analysis because of its simplicity and the availa-
bility of equipment.  However this method is not specific enough to
distinguish $VB_{12}$ from its various non-biologically active analogs.  Thus
spectrophotometric measurements had to be checked by a microbiological
assay (85).

In order to determine the $VB_{12}$ content of the cells, the cells
were removed from the fermentation broth by centrifuging at 2500 g's
for 15 minutes.  After washing with distilled water the cells were broken
by heating or addition of acetone.  In measurements of the $VB_{12}$ content
of the cell free broth or of the broth including the cells, corrections
had to be made for the effects of spent liquor components on the spectro-
photometric measurements.

The spectrophotometric technique was a modification of the
method described by Rudkin and Taylor (93) and Fisher (94).

As previously noted there are several types of cobalamins
(hydroxo, chloro, nitro, etc.).  These can readily be converted to

cyanocobalamin by treatment with cyanide ions. In an alkaline solution containing an excess of cyanide ion cyanocobalamin takes on a second cyanide group to form a dicyanide cobalamin (purple coloured complex) which is unstable, existing only in an excess of cyanide ion. The colorimetric assays of Rudkin and Taylor, and Fisher are based on the difference in the visible absorption spectra of cyanocobalamin and its dicyanide complex formed in an excess of cyanide ion.

Some of the components of the sulfite spent liquor tend to interfere with this analytical procedure.

$VB_{12}$ analysis according to Rudkin and Taylor is as follows:

"1. In a well ventilated hood solid sodium cyanide is added to a sample of the unknown containing approximately 200 micrograms of $VB_{12}$, at a concentration of not less than 1 microgram per ml, so that the final concentration of cyanide is 1%. The sample is stirred to dissolve the sodium cyanide and the pH is adjusted to 9.5 - 10.0 with 10% sodium hydroxide solution if necessary.

2. This is allowed to stand for 5 hours at room temperature for complete conversion of the $VB_{12}$ to the dicyanide complex.

3. Solid sodium sulfate (20% w./v.) is added and dissolved. The pH is further adjusted with sodium hydroxide to pH 11.0 to 11.5 and the aqueous solution is extracted three times with one tenth volume of benzyl alcohol.

4. To the combined benzyl alcohol extracts one-half volume of chloroform is added and the solvent phase is extracted three times with one-tenth volume of water. The aqueous phase is made up to 25 ml.

5. To a 10 ml aliquot of the water extract, 2 ml of a 10% sodium cyanide solution is added. To another 10 ml aliquot, 2 ml of a 12.5% potassium dihydrogen phosphate solution is added to adjust the pH to 5 to 6.

6. The optical density of each solution is measured at 582 m$\mu$.

7. The $VB_{12}$ amount is calculated from the difference, $(\Delta E)$obsd, in the optical densities at 582 m$\mu$. $\Delta E_{1cm}^{1\%} = 54$ for crystalline $VB_{12}$.

8. Total $VB_{12}$ activity in sample

$$= \frac{(\Delta E)\text{obsd.} \times 6/5 \times 25 \times 1.03}{0.0054 \times (\text{cm cell length})} \qquad \text{eq (1)}$$

where 1.03 is an extraction factor."

Spectra for cyanocobalamin and its dicyanide complex are shown in Fig. 4. These are for pure $VB_{12}$ purchased from the British Drug Houses Ltd. Note that spectra intersect at 524, 554 and 650 m$\mu$. The spectra of sulfite spent liquor containing 200 $\mu$g $VB_{12}$ per sample is shown in Fig. 5. Also the absorption spectra of sulfite spent liquor are shown in Fig. 6.

Due to interference of the spent liquor, the optical density difference at 582 m$\mu$, $\Delta E_{582}$ in Fig. 5, cannot be used as $(\Delta E)$obsd. to calculate $VB_{12}$ activity. $\Delta E$ at 582 m$\mu$ due to the spent liquor, $\Delta E_s$, can be calculated according to Beer's law by interpolation of $\Delta E_{524}$, $\Delta E_{554}$ and $\Delta E_{650}$ as these are due to spent liquor only. In the case of Fig. 5 $\Delta E_{524} = 0.050$, $\Delta E_{554} = 0.036$, $\Delta E_{582} = 0.059$, and $\Delta E_{650} = 0.016$. The optical density difference at 582 m$\mu$ due to spent liquor is calculated to be 0.029. Thus $(\Delta E)$obsd. $= \Delta E_{582} - \Delta E_s = 0.059 - 0.029 = 0.030$. From eq. (1) the $VB_{12}$ activity is calculated to be 173 $\mu$g, the actual value was 200 $\mu$g.

Figure 4

SPECTRA OF VB₁₂

Dicyanide complex

Cyanocobalamin

OPTICAL DENSITY

WAVE LENGTH ( mµ )

Figure 5 200 µg VB₁₂ IN SSL

Figure 6
SPECTRA OF SSL
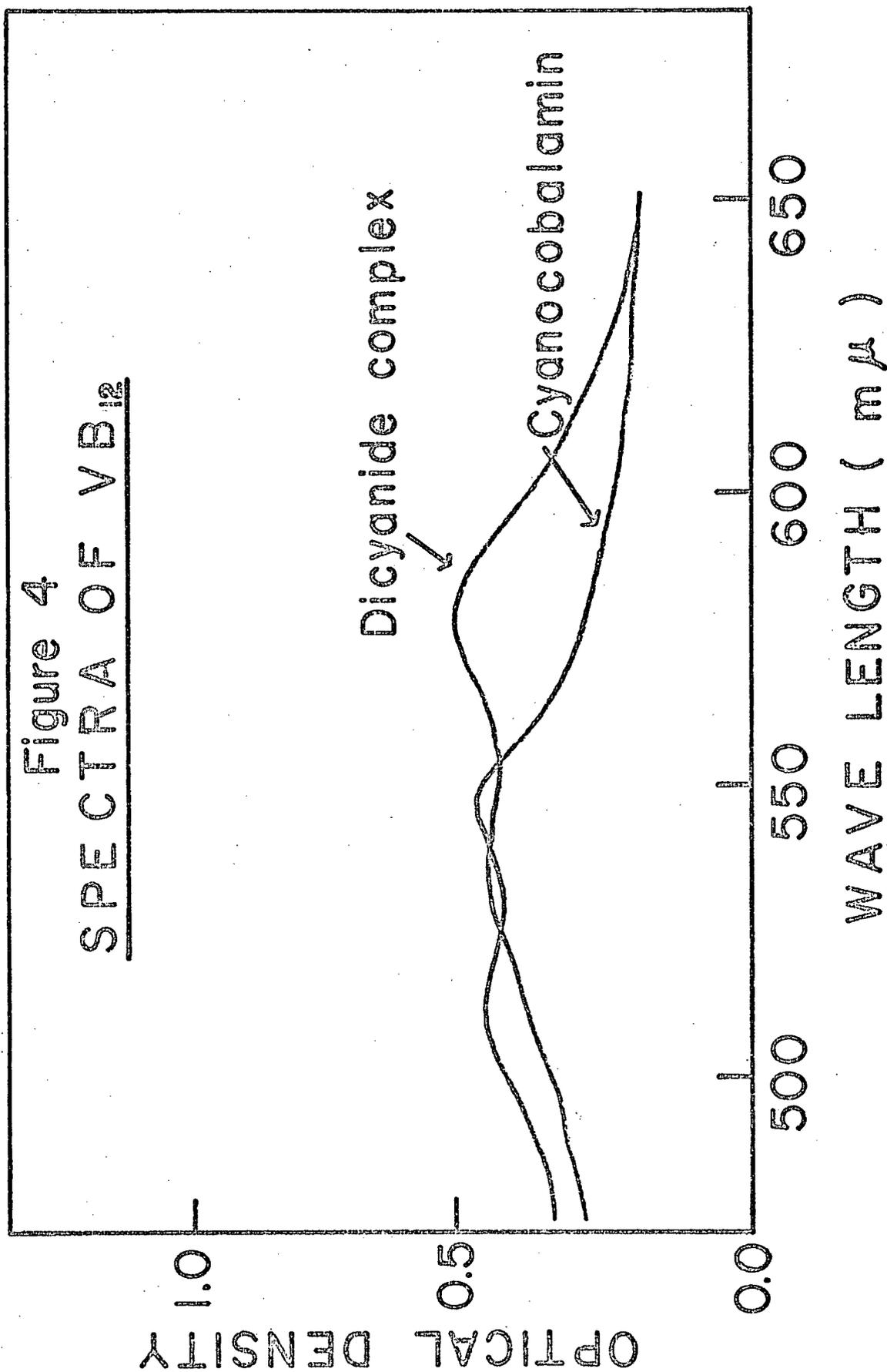
The microbiological assay used Lactobacillus leichmanii,
(ATCC 7830) as the test organism. This is a 16 to 24 hour turbidimetric
procedure based on the U.S.P. XVI method (84). This is done as follows:

1. To five test tubes (approx. 20 x 150 mm) add 1.0, 2.0, 3,0,
4.0 and 5.0 ml respectively of the cyanocobalamin standard reference
solution. This is done in triplicate. Now sufficient distilled water to
make up each volume to 5.0 ml is added.

2. To another series of tubes, the same amount of the test
solution of the material to be assayed is added in the same manner as
outlined in step 1, again in triplicate. Then sufficient distilled water
to make 5.0 ml is added.

3. Another 3 test tubes with 5.0 ml of distilled water are
prepared as blanks.

4. To each tube 5.0 ml of basal medium stock solution (Bacto-
Difco $B_{12}$ assay medium U.S.P. No. 0541-15) is mixed.

5. Aseptically, one drop of inoculum is added to each tube
except one of the 3 blank tubes. Tube contents are mixed and incubated
at 37°C for 16-24 hours. There should be no substantial increase in
turbidity in the tubes containing the highest level of standard during
a 2 hour period.

6. The turbidity of the cultures is read in a suitable photo-
metric instrument at a specific wavelength between 540 and 660 mμ. In
making readings, the contents of each tube should be thoroughly mixed.
After mixing tube contents are transferred to optical glassware, and the tur-
bidity is read.

7. Using the uninoculated blank, the meter of the instrument is

set to read 100% transmittancy, then the transmittancy of the inoculated

blank is read. If the inoculated blank tubes give a transmittancy of

less than 65%, this indicates interference due to $VB_{12}$ activity in the

basal medium stock solution, or the inoculum.

8. Using the inoculated blank and standard specimen, a

standard response curve is prepared by plotting the % transmittancy

reading for each level of standard cyanocabalamin solution used. A

smooth curve should be drawn through the plotted points. One of standard

curves which was made in this experiment is shown in Fig. 7.

9. Using this standard curve, by interpolation the amount of

cyanocobalamin equivalent to the $VB_{12}$ activity of each ml of the test sample

under assay is determined.

## 2-7 Sulfite spent liquor preparation

Calcium and ammonium base sulfite spent liquors were obtained

from the pilot plant digesters of Columbia Cellulose Research and Develop-

ment Division. The analysis of these liquors is presented in Table 3.

Raw sulfite spent liquor contains much residual $SO_2$ from the

pulp cook. A large proportion of loosely bound $SO_2$ is believed to be

attached to the wood sugars in the form of 2-hydroxy sulfonate as shown

in Fig. 8. Some $SO_2$ may merely be dissolved in the spent liquor. As 200 ppm

of $SO_2$ is sufficient to sterilize the liquor against bacteria (72), the $SO_2$

concentration must be reduced below this level. $SO_2$ removal was brought

about by adjusting the pH of the liquor to about 5.5 with $CaCO_3$. The

precipitate which resulted was removed by filtration. Finally, nutrients

were added; these were yeast extract (1% by weight), cobalt chloride (5 ppm),

Figure 7

MICROBIOLOGICAL ASSAY
STANDARD CURVE

Table   3

Typical Analysis of Sulfite Spent Liquor
from Columbia Cellulose (Ca base)

| PH | 1.6 |
|---|---|
| Total solids | 128 gr/l |
| Total sugars | 37.4 gr/l |
| Fermentable sugars | 64% of total sugars |
| Lignosulfonate | 79.3 gr/l |

The Composition of a Spruce Sulfite Spent Liquor by Forss (9)

| Total solids | 12 - 16% of SSL |
|---|---|
| Lignosulfonate | 52% of total solids |
| Monosaccharides | 23%        " |
| Poly and Oligo saccharides | 6%        " |
| Organic acids | 7%        " |
| Calcium | 5%        " |
| Sugar sulfonate | 3%        " |
| Others | 4%        " |

# Figure 8

## SUGAR--BISULFITE
## COMPLEX
## IN SSL

$$RCHO + HSO_3^- \rightleftharpoons RC{\overset{\displaystyle H}{\underset{\displaystyle OH}{\phantom{-}}}}SO_3^-$$

α-Hydroxysulfonate

( loosely bound $SO_2$ )

and potassium dihydrogen orthophosphate (0.4% by weight). This solution was then tested for its ability to support bacterial growth of P. freudenreichii, and it is referred to as "non-treated" medium.

"Treated" medium was prepared by taking non-treated medium before the addition of nutrients and adjusting its pH to 12 with $Ca(OH)_2$. This caused further precipitation which was assumed to be calciumligno-sulfonate (73). The precipitate was filtered off, the pH readjusted to between 6 and 7 with $H_2SO_4$, and nutrients were added as for the non-treated medium. Growth tests were then performed. When the pH of the liquor was held at 12, more and more precipitate was formed. Since the precipitate contains a certain amount of wood sugars, the sugar content of the treated media could be roughly adjusted at this stage in the treatment by allowing more or less precipitate to form. Precipitation could be accelerated by heating. Since the lignin compounds are mainly responsible for the colour of the sulfite spent liquor (74, 75), the treated medium was much clearer than the non-treated medium.

Elements such as iron, zinc, and copper which are essential for bacterial growth were assumed to be present in small quantities in sulfite spent liquor and were therefore not added.

The addition of cobalt chloride to the media must be carefully controlled since it has been reported that a cobalt level above 50 ppm inhibits the synthesis of $VB_{12}$ (39, 76, 77, 78).

2-8 Synthetic media preparation

For comparison with the sulfite spent liquor base media, the same amount of nutrient as in a treated and non-treated media and 1%

glucose and a little calcium carbonate (less than 0.05%) were added to distilled water and is referred to as synthetic medium. This medium was also used to keep the culture.

## 2-9 Fermentation technique

For the inoculum, P. freudenreichii from a synthetic medium containing 1% beef extract, 0.3% yeast extract, 1% peptone, 0.5% dextrose, 0.1% soluble starch, 0.05% cysteine hydrochloride, 0.5% sodium chloride, 0.3% sodium acetate, and 0.05% agar was transferred 24 hours after its inoculation from tomato juice agar to the treated or non-treated medium in a test tube. After several transfers from test tube to test tube the bacteria changed its shape to cocci as described before and showed good growth in treated media.

0.5 ml of culture was used to inoculate every 5 ml of fermentation medium.

## 2-9-1 Sterilization

Sterilization of media was carried out in an autoclave at 120°C. Depending on the volume of media, the sterilization time was varied from 10 minutes to 45 minutes.

## 2-9-2 Test tube growth test

Test tube growth tests were carried out using cotton plugged, stationary test tubes. To measure the bacterial growth in the test tubes, test tubes for the spectronic 20 were used and turbidity was measured 48 hours to 120 hours after inoculation. In these tests the media were not diluted before turbidity readings were made.

## 2-9-3  Bellco glass fermentor

The type of glass fermentors used in this experiments were Bellco glass fermentors (100 ml and 500 ml).  The fermentation temperature was $30 \pm 0.5°C$.  Agitation speed was 0.5 - 4 rps.  Sampling was done using a sterilized pipet through a sample port which was plugged with cotton.  The pH was not adjusted during these experiments since pH was not considered to be an important factor if the initial pH was between 5.5 and 7.  All fermentations were done with treated calcium base spent liquor media.

## 2-9-4  7 ℓ fermentation

This was carried out using a 7ℓ fermentor (Bio-Kulture; Fermentation Design INC.).  The diagram of this fermentor is shown in Fig. 9.. The fermentation temperature was controlled at $30°C$, agitation speed was about 3 rps; the pH was not controlled.  As noted before in section 1-3, only agitation was employed, aeration was not employed.  Sampling was done using a sterilized pipet.  The cell bodies were collected with a Sharples super centrifuge after fermentation.

## 3  Results

### 3-1  Spectrophotometric analysis for $VB_{12}$

The results of a check on the modified spectrophotometric technique for measuring $VB_{12}$ in the presence of sulfite spent liquor are presented in Table 4 and Fig. 10.  Comparison of measured amounts of $VB_{12}$ with the amounts added indicates reasonable agreement.

### 3-2  Comparison of spectrophotometric and microbiological methods

The spectrophotometric and microbiological $VB_{12}$ assays are

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Figure 9    7L JAR-FERMENTOR

Table 4

Test of Accuracy of the Spectrophotometric Method
for $VB_{12}$ Analysis

| Actual $VB_{12}$ content (µg) | Measured $VB_{12}$ content (µg) |
| --- | --- |
| 400 | 433 |
| 300 | 254 |
| 200 | 152 |
| 200 | 206 |
| 48 | 25 |
| 0 | -6 |
| 169 | 154 |
| 169 | 161 |
| 0 | -5 |
| 169 | 158 |
| 200 | 175 |
| 169 | 191 |
| 169 | 185 |
| 169 | 175 |
| 0 | 0 |

Figure 10

TEST OF SPECTROPHOTOMETRIC ASSAY

compared in Table 5 and Fig. 11. Agreement can be said to be reasonable.

3-3  Test tube growth tests.

As shown in Table 6 and 7 no bacterial growth could be detected in the non-treated spent liquor medium. The only times that growth was observed in the non-treated medium were when sufficient synthetic medium was added with the inoculum that the bacteria could grow on it.

In treated sulfite spent liquor medium good growth was observed as shown in Table 7 and Fig. 12.

Without yeast extract no growth was observed in the calcium base liquor. This of course indicates that some external source of nitrogen is necessary. The fact that only slight growth was observed in ammonia base liquor in the absence of yeast extract indicates the need for a nitrogen source other than ammonia. Reasonable growth was observed in the ammonia base liquor after the addition of yeast extract.

3-4  Bellco glass fermentor and 7ℓ fermentor

Results obtained from a number of fermentations in Bellco glass fermentors (250 ml and 500 ml) with treated sulfite spent liquor medium are recorded in Tables 8 to 10. Fig. 13 is a graphical presentation of the results of one of these fermentations.

Similar data for 7 liter fermentations with both synthetic and treated calcium base spent liquor media are provided in Table 11 and Figs. 14 and 15.

Table 5

Comparison of Microbiological and Spectrophotometric Assay

| Sample | $VB_{12}$ activity (µg/1) Spectrophotometric assay | $VB_{12}$ activity (µg/1) Microbiological assay |
|---|---|---|
| SSL small fermentor (500 ml) | 210 380 590 860 830 | 220 530 760 1100 960 |
| Synthetic 7 ℓ fermentor | 320 480 930 2050 | 350 500 780 1620 |
| SSL 7 ℓ fermentor | -30 120 210 195 333 480 | 75 135 113 225 465 650 |

Figure II    COMPARISON  OF ASSAY

Table  6

Test Tube Growth Test in Non-Treated Liquor

| Base | Dilution ratio | pH at Start | Yeast (%) | Peptone (%) | Growth |
|------|----------------|-------------|-----------|-------------|--------|
| Ca | undiluted | 5 | 1.0 | 0 | No |
| | " | 5 | 0 | 1.0 | No |
| | " | 5 | 0 | 0 | No |
| | " | 5 | 0.1 | 1.0 | No |
| | " | 6 | 0.1 | 1.0 | No |
| | " | 7 | 0.1 | 1.0 | No |
| | 12.5  S | 6.2 | 1.0 | 0 | Yes |
| | 4 | 6.3 | 1.0 | 0 | Yes |
| | 5 | 5.5 | 1.0 | 0 | No |
| | 3 | 5.5 | 1.0 | 0 | No |
| | 2 | 5.5 | 1.0 | 0 | No |
| | undiluted | 5.5 | 1.0 | 0 | No |
| NH$_3$ | undiluted | 5.3 | 1.0 | 0 | No |
| | " | 6.1 | 1.0 | 0 | No |
| | 2 | | 1.0 | 0 | No |
| | 2 | | 0 | 0 | No |
| | 4 | | 1.0 | 0 | No |
| | 4 | | 0 | 0 | No |
| | undiluted | 6.1 | 0 | 0 | No |
| | 5 | | 1.0 | 0 | No |
| | 3 | | 1.0 | 0 | No |
| | 2 | | 1.0 | 0 | No |

$$\text{Dilution ratio with S} = \frac{\text{Synthetic media + SSL media}}{\text{SSL media}}$$

$$\text{Dilution ratio} = \frac{\text{Distilled water + SSL media}}{\text{SSL media}}$$

Table 7

Test Tube Growth Test in Treated Liquor

| Base | Dilution ratio | Sugar (g/l) | pH | Yeast (%) | Growth |
|------|----------------|-------------|-----|-----------|--------|
| Ca | No dilution | 8.6 | 5.0 | 1.0 | good |
| | No dilution | 15.1 | 5.0 | 1.0 | good |
| | No dilution | 15.1 | 6.8 | 1.0 | good |
| | 2 | 7.6 | | 1.0 | good |
| | 2 | 7.6 | | 0 | none |
| | 4 | 3.8 | | 1.0 | fair |
| | 4 | 3.8 | | 0 | none |
| | No dilution | 8.6 | 7.0 | 1.0 | good |
| | No dilution | H* | 5.1 | 1.0 | fair |
| | No dilution | 9.1 | 6.3 | 1.0 | good |
| | 2 | 4.6 | | 1.0 | good |
| | No dilution | H | 7.0 | 1.0 | fair |
| | 2 | H | | 1.0 | poor |
| NH$_3$ | No dilution | 14.7 | 6.1 | 1.0 | poor |
| | 2 | 7.4 | 6.0 | 1.0 | fair |
| | No dilution | 14.7 | 6.3 | 1.0 | good |
| | No dilution | 14.7 | 6.3 | 0 | poor |
| | 2 | 7.4 | | 1.0 | good |
| | 2 | 7.4 | | 0 | none |
| | 4 | 3.7 | | 1.0 | fair |
| | 4 | 3.7 | | 0 | none |
| | No dilution | 14.7 | 7.1 | 1.0 | fair |
| | No dilution | H | 7.3 | 1.0 | fair |
| | No dilution | | 6.6 | 1.0 | fair |
| | 2 | | | 1.0 | poor |
| | No dilution | H | 6.3 | 1.0 | fair |
| | 2 | H | | 1.0 | poor |

*H - Heated during precipitation of lignin.

# Figure 12

## TEST TUBE GROWTH TEST

### ( TREATED SSL )

Table   8

Bellco Glass Fermentor

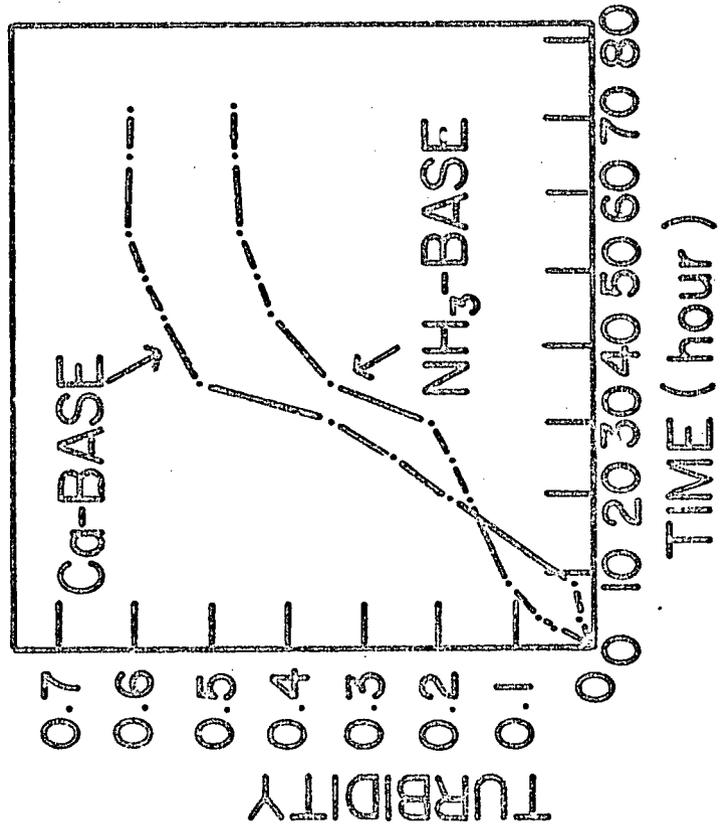| Volume of fermentor (ml) | Fermentation time (hr) | Sugar (g/l) | pH | COD (mg/l) | Acids (m mole/l) | $VB_{12}$ (mg/l) | Cell weight (g/l) |
|---|---|---|---|---|---|---|---|
| 100 | 0 | 2.14 | 6.1 | 53,000 | | | |
|  | 118 | 0.99 | 4.9 | 49,000 | 44.7 | 0.7 | |
| 500 | 0 | 3.7 | 5.8 | 71,000 | | | |
|  | 118 | 0.9 | 5.0 | 53,500 | | 1.03 | 2.04 |
| 100 | 0 | 2.5 | 5.5 | 60,700 | | | |
|  | 168 | 0.4 | 4.9 | 52,000 | 53.1 | | |
| 500 | 0 | 7.5 | 5.7 | 68,000 | | | |
|  | 168 | 3.2 | 5.0 | 52,000 | | | |
| 100 | 0 | 7.5 | 5.75 | 61,120 | | | |
|  | 120 | 5.9 | 4.6 | 56,700 | | 0.6 | 1.47 |
| 500 | 0 | 9.5 | 5.92 | 69,840 | | | |
|  | 120 | 7.7 | 4.7 | 63,800 | | 0.8 | 1.65 |
| 500 No agitation | 0 | 3.6 | 5.90 | 57,640 | | | |
|  | 120 | 1.9 | 4.90 | 50,300 | | 0.3 | 0.84 |
| 100 | 0 | 6.7 | 5.9 | 60,600 | | | |
|  | 79 | 4.6 | 5.1 | 57,900 | | | |
|  | 100 | 4.0 | 4.9 | 57,100 | | | |
|  | 124 | 3.5 | 4.7 | 50,600 | | 0.11 | 0.55 |
| 100 | 0 | 8.59 | 6.3 | 62,800 | | | |
|  | 84 | 1.61 | 5.0 | 46,800 | | 0.47 | |

Table 9

Bellco Glass Fermentor

| Volume of fermentor (ml) | Fermentation time(hr) | Sugar (g/l) | pH | COD (mg/l) | Acids (m mole/l) | VB$_{12}$ (mg/l) | Turbidity |
|---|---|---|---|---|---|---|---|
| 500 | 0 | 6.7 | 5.75 | 59,700 | | | |
| | 80 | 4.5 | 5.00 | 57,900 | 33 | | 0.075 |
| | 100 | 4.0 | 4.80 | 57,100 | | | 0.46 |
| | 124 | 3.6 | 4.80 | 50,600 | 41 | | 0.51 |
| | 148 | 2.7 | 4.80 | 46,300 | | | 0.48 |
| | 172 | 0.4 | 4.75 | 44,000 | 53 | 0.81 | 0.81 |
| | Cell weight 2.28 g/l | | | | | | |
| 500 | 0 | 10.8 | 5.8 | 73,800 | | | |
| | 24 | 11.2 | 5.52 | 77,300 | 0.5 | 0.2 | 0.06 |
| | 35 | 10.4 | 5.22 | 74,300 | 14.1 | | 0.185 |
| | 48 | 9.9 | 5.05 | 72,000 | 32.7 | 0.34 | 0.377 |
| | 72 | 8.1 | 4.88 | 72,000 | 51.9 | | 0.605 |
| | 120 | 6.9 | 4.83 | 70,500 | 59.0 | 0.82 | 0.73 |
| | 168 | 5.9 | 4.77 | 68,500 | 73.2 | 1.41 | 0.82 |
| | Cell weight 2.83 g/l | | | | | | |
| 100 | 0 | 12.1 | 6.4 | 73,100 | | | |
| | 12 | 12.1 | 6.2 | | 3.5 | | 0.03 |
| | 24 | 11.3 | 5.9 | | 7.3 | | 0.07 |
| | 48 | 10.1 | 5.1 | | 34.2 | | 0.39 |
| | 72 | 7.6 | 4.75 | | 68.3 | | 0.61 |
| | 120 | 6.2 | 4.7 | | 81.3 | | 0.70 |
| | 168 | 5.12 | 4.7 | 58,000 | 87.5 | 1.05 | 0.73 |
| | Cell weight 2.81 g/l | | | | | | |
| 500 | 0 | 6.30 | 6.61 | 73,500 | | | |
| | 12 | 6.39 | 6.03 | | 0.10 | | 0.02 |
| | 24 | 6.26 | 5.87 | | 0.40 | | 0.14 |
| | 48 | 5.36 | 5.10 | | 21.5 | | 0.29 |
| | 72 | 4.12 | 4.98 | | 29.3 | | 0.40 |
| | 120 | 2.29 | 4.88 | | 32.5 | | 0.43 |
| | 168 | 1.15 | 4.83 | 54,800 | 36.0 | 0.85 | 0.46 |
| | Cell weight 2.19 g/l | | | | | | |

Table 10

Bellco Glass Fermentor

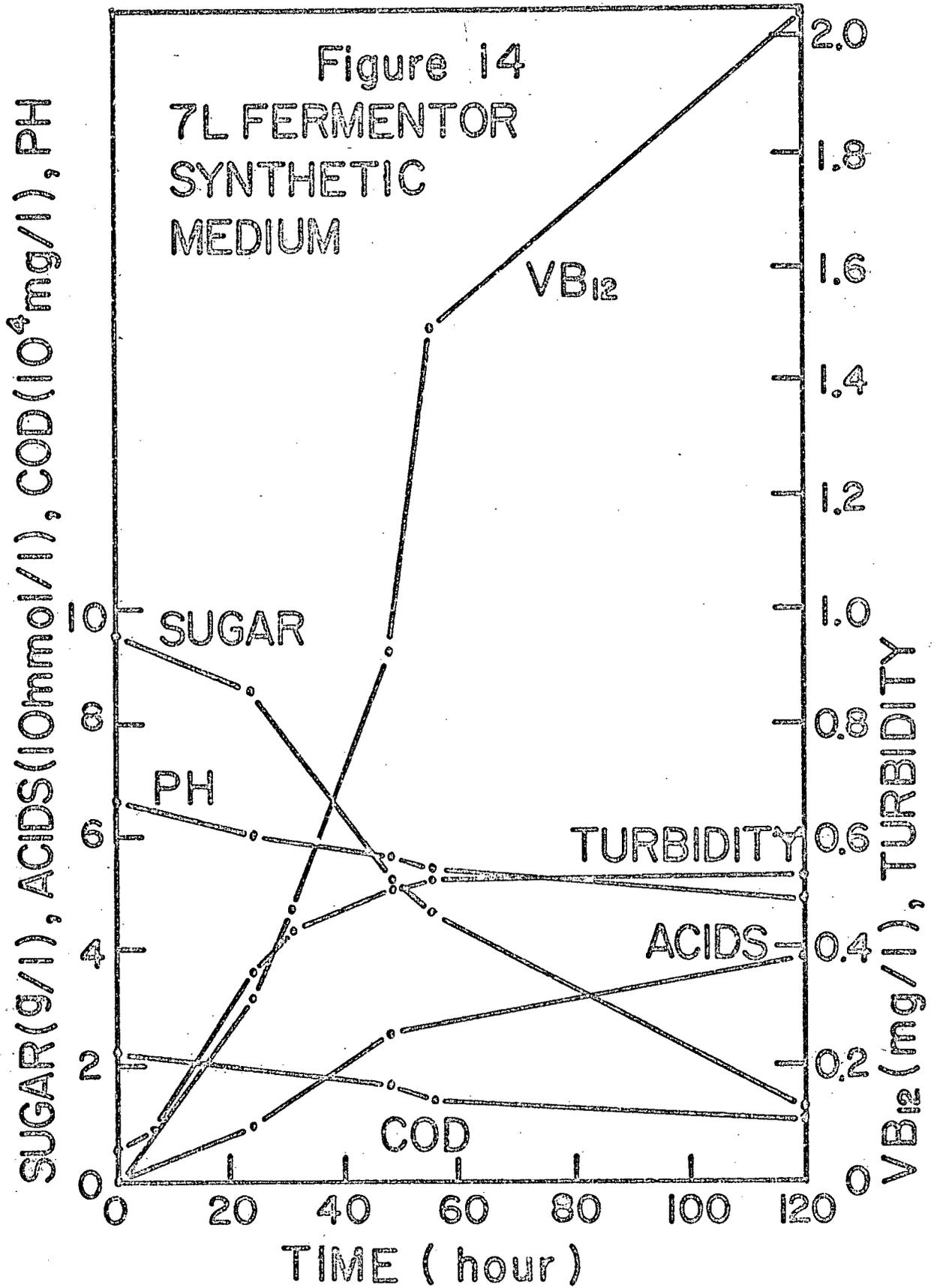| Volume of fermentor (ml) | Fermen- tation time(hr) | Sugar (g/l) | pH | COD (mg/l) | Acids (m mole/l) | $VB_{12}$ (mg/l) | Turbi- dity |
|---|---|---|---|---|---|---|---|
| 500 | 0 | 10.9 | 5.87 | 79,400 | | | 0.021 |
| | 12 | 9.80 | 5.41 | 62,500 | 2.4 | | 0.072 |
| | 24 | 9.01 | 5.23 | 58,100 | 8.5 | 0.21 | 0.140 |
| | 48 | 6.78 | 5.11 | 54,600 | 23.6 | 0.38 | 0.247 |
| | 72 | 4.73 | 5.00 | 46,600 | 32.4 | 0.59 | 0.383 |
| | 96 | 3.81 | 4.85 | 46,800 | 34.9 | 0.86 | 0.515 |
| | 120 | 2.85 | 4.83 | 46,200 | 36.3 | 0.83 | 0.521 |
| | | | | Cell weight 1.67 g/l | | | |

Figure 13  500 ML BELLCO FERMENTOR SSL MEDIUM

Table 11

7ℓ Fermentor Fermentations

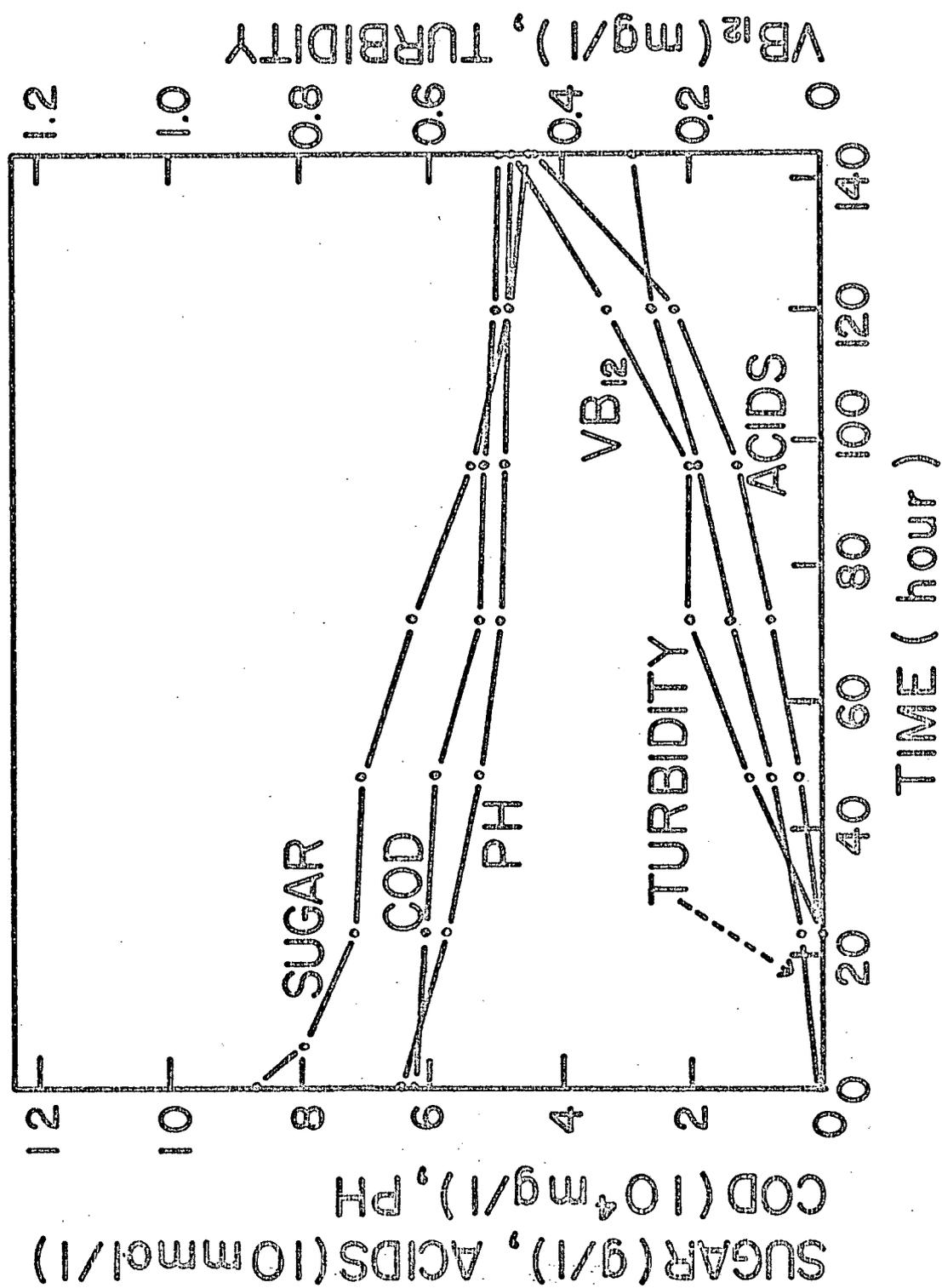| Medium | Time (hr) | Sugar (g/1) | pH | COD | Acids (m mole/1) | VB$_{12}$ (mg/1) | Turbidity |
|---|---|---|---|---|---|---|---|
| SSL | 0 | 8.80 | 6.50 | 63,000 | 0 | 0 | 0.021 |
| | 6 | 8.00 | | 62,700 | 0 | | |
| | 24 | 7.20 | 5,80 | 61,300 | 0.3 | 0 | 0.041 |
| | 48 | 7.10 | 5.30 | 59,800 | 4.4 | 0.12 | 0.082 |
| | 72 | 6.35 | 5.00 | 53,000 | 8.1 | 0.21 | 0.148 |
| | 96 | 5.40 | 4.85 | 52,300 | 13.6 | 0.195 | 0.196 |
| | 120 | 4.83 | 4.80 | 50,800 | 22.8 | 0.333 | 0.264 |
| | 144 | 4.52 | 4.81 | 49,500 | 44.3 | 0.48 | 0.296 |
| | Cell weight 1.05 g/1 | | | | | | |
| Synthetic | 0 | 9.60 | 6.7 | 22,800 | 0 | 0 | 0.053 |
| | 7 | | | | | | 0.095 |
| | 24 | 8.60 | 5.75 | | 10.0 | 0.32 | 0.364 |
| | 31 | | | | | 0.48 | 0.445 |
| | 48 | 5.24 | 5.03 | 17,600 | 26.3 | 0.93 | 0.512 |
| | 55 | 4.67 | 4.85 | 14,700 | | 1.50 | 0.533 |
| | 120 | 1.24 | 4.50 | 11,900 | 40.5 | 2.05 | 0.540 |
| | Cell weight 2.37 g/1 | | | | | | |

Figure 14
7L FERMENTOR
SYNTHETIC
MEDIUM

Figure 15 7L FERMENTOR SSL MEDIUM

Table 12

Volatile Acids

| Sugar used (g/l) | Volatile Acids (g/l) | Degree of conversion* (%) |
|---|---|---|
| 1.15 | 3.04 | 264 |
| 2.10 | 3.71 | 176 |
| 6.98 | 6.12 | 88 |
| 5.15 | 2.52 | 49 |
| 6.30 | 3.73 | 59 |
| 4.90 | 5.13 | 104 |
| 8.04 | 2.68 | 34 |
| 8.36 | 2.84 | 34 |
| 4.28 | 3.10 | 72 |

\* Average mol weight is calculated according to the result that the ratio between propionic and acetic acid is roughly 2 : 1.

### 3-4-1 COD

Results indicate that for all the fermentations analyzed, the COD value was reduced by fermentation. However the COD of the media after fermentation is still high indicating that compounds other than fermentable sugars are responsible for most of the COD of the treated spent liquor after fermentation. A COD vs. sugar content graph is shown in Fig. 16.

### 3-4-2 Volatile acids

From Figs. 10, 12, and 13 one can see from the general shape of the curves of volatile acids production and turbidity vs. time that acids production seems to closely follow cell production.

### 3-4-3 $VB_{12}$

Figs. 10, 12, and 13 show that $VB_{12}$ content of the media, including the cells, increases with time. In one curve a falling off was noted after 100 hours (Fig. 10). Much higher levels of $VB_{12}$ concentration were achieved in the synthetic media.

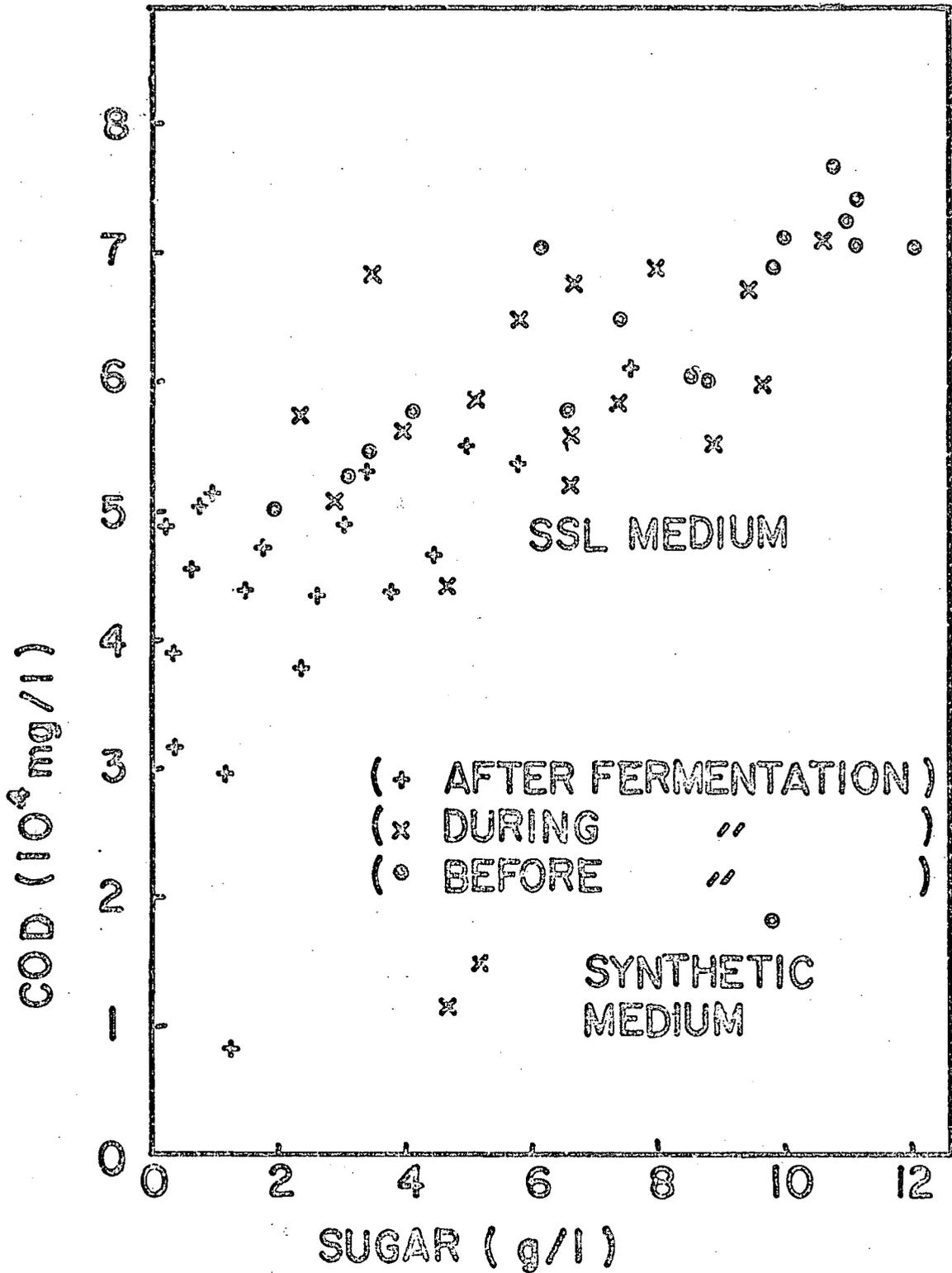Distribution of $VB_{12}$ activity in the cells and in the cell free media is shown in Table 13 for the 7ℓ fermentations.

Figure 16   COD vs. SUGAR CONTENT

Table 13

VB$_{12}$ Activity in 7ℓ Fermentation

(By L. leichmanii method)

| Medium | Whole broth VB$_{12}$ (mg/1) | Medium liquor VB$_{12}$ (mg/1) | Cell body VB$_{12}$ (mg/g dry cell) | Cell growth (g/1) | Calculated total |
|---|---|---|---|---|---|
| SSL | 0.65 | 0.213 | 0.444 | 1.05 | 0.679 |
| Synthetic | 1.62 | 0.275 | 0.463 | 2.37 | 1.37 |

4. Discussion

The spectrophotometric method requires less time than the microbiological assay if only a few samples are to be analyzed. However the microbiological assay lends itself to the determination of the $VB_{12}$ content of a large number of samples. It has the further advantage of requiring a much smaller sample volume. If the required equipment is available the microbiological assay is to be preferred since this method is specific for biologically active $VB_{12}$.

During the extraction of the $VB_{12}$ from the spent liquor with benzyl alcohol in the spectrophotometric assay, foaming of the spent liquor interferes, especially with non-treated liquor. Sometimes this foam did not disappear after 24 hours; these samples were discarded as the dicyanide complex of $VB_{12}$ is not stable after 24 hours. This interference due to foaming at the liquid-liquid interface may interfere with the extraction factor used by Rudkin and Taylor (93) in calculating the $VB_{12}$ content. Note that in Fig. 10 the best line through the measured results falls somewhat below the expected line. This foaming problem does not arise in the microbiological assay nor for that matter was foaming a problem in the larger scale fermentations.

Although the microbiological assay is preferable it involves a great deal of tedious pipetting. The spectrophotometric assay is reasonably easy to do and gives an acceptable result as evidenced by Table 4. Chaiet et.al. (79) noted quite different results in measuring the $VB_{12}$ content of animal protein factor (APF) by each of these techniques; we encountered no such discrepancies. While there is some scatter evident

in Fig. 11, the microbiological assay does not give results consistently
higher than, or lower than the spectrophotometric assay results as
was noted in Fig. 11.

In the untreated medium growth was not observed, hence we
assume that as good growth occurs in the treated medium, the lack of
growth in the untreated medium must have been due to the presence of
toxic agents (eg. $SO_2$) which were removed in the lignosulfonic acid precipita-
tion stage.

Growth in ammonia base, treated media was not as good as in
calcium base, treated media. The reason for this is not known with
certainty but we may speculate that a lignin condensation reaction (75),
variations in the polysaccharide-monosaccharide ratio, or variations in
the sulfur compounds may be responsible.

Batch fermentations usually follow a certain sequence. First
there is a lag phase in which the bacteria adapt themselves to the medium.
Cell mass increases but little or no cell division occurs. This is
followed by a log phase in which the specific growth rate is constant.
Then a stationary phase is observed in which the cells are alive but not
dividing due to the lack of some nutrient or the presence of some toxic
factor. Finally there is a decline or death phase wherein the cells die.
This behaviour occurs when a single nutritional factor is limiting. The
growth, as measured by turbidity of P. freudenreichii in a synthetic media,
which contains only a single sugar, follows this pattern. This can be
seen in Fig. 14. Perhaps this is more clearly shown in Fig. 17 where
the volumetric rates are plotted against time. Fig. 17 was derived from

Fig. 14.  Note that there is only a single peak in the turbidity curve

and in the sugar comsumption curve.

When more than one sugar is available for the metabolic pro-

cesses of the microorganism it may use one preferentially.  When the

supply of this sugar is exhausted the organism adapts itself to using

another.  However this may involve another lag period.  This sort of

behaviour can be seen in Figs. 12, 13, 15, 18, and 19.  In the rate curves

(Figs. 18 and 19) the sugar comsumption rate curves have two peaks as

do the turbidity curves.  This may be due to the preferential utilization

of one type of sugar by the bacteria; perhaps hexoses are used before

pentoses.

The long lag phase noted in Fig. 15 may be due to the fact

that the bacteria in the inoculum were not properly adapted to the spent

liquor.  A new batch of sulfite spent liquor was used in this experiment.

The volumetric basis fermentation rate curves (Figs. 17, 18 and

19) show how the rates of production of cells, $VB_{12}$ and organic acid

production vary with time.  They also show the rate of substrate consump-

tion.  In the synthetic media the growth curve appears to follow the sugar

consumption curve and the product curves ($VB_{12}$ and organic acids) seem

to follow the growth (turbidity) curve.

In the sulfite spent liquor medium the picture is not quite

so clear.  The rate of production of acid and $VB_{12}$ follow the growth (tur-

bidity) curve but the sugar consumption and growth curves do not show

trends similar to those observed with the synthetic media.  As was men-

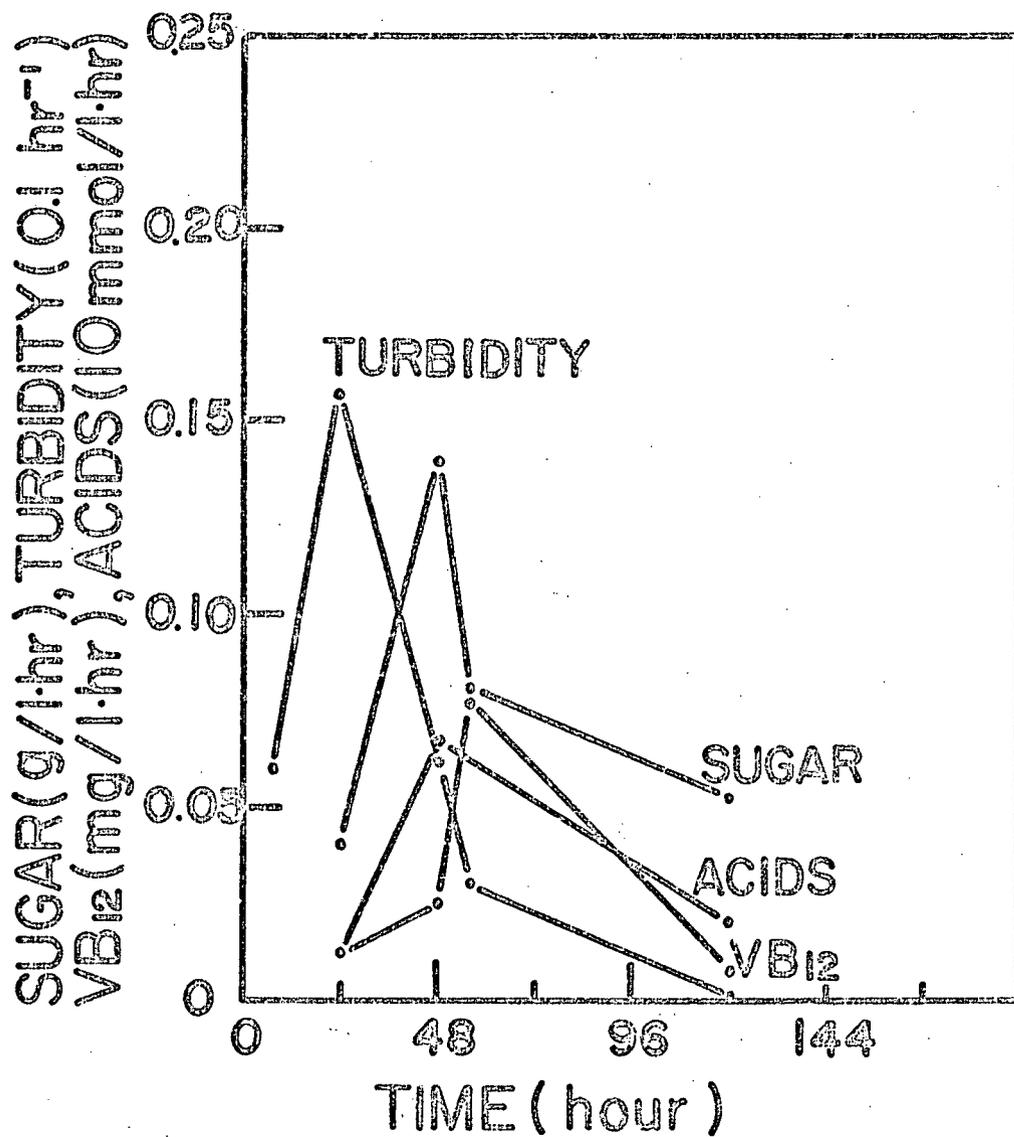tioned earlier, the bacteria have the choice of a number of sugars in

Figure 17    FERMENTATION RATE
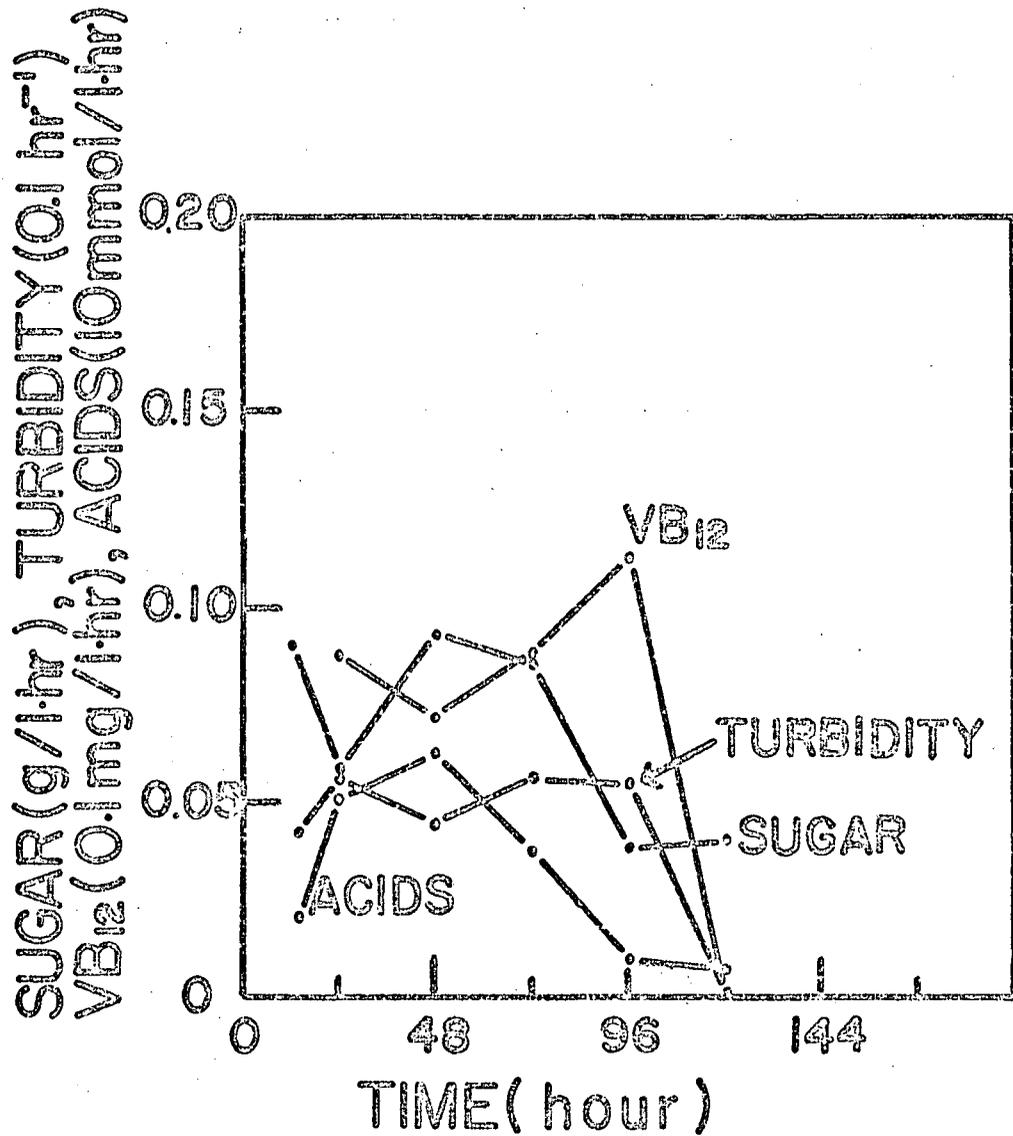7L FERMENTOR
SYNTHETIC MEDIUM

Figure 18    FERMENTATION RATE
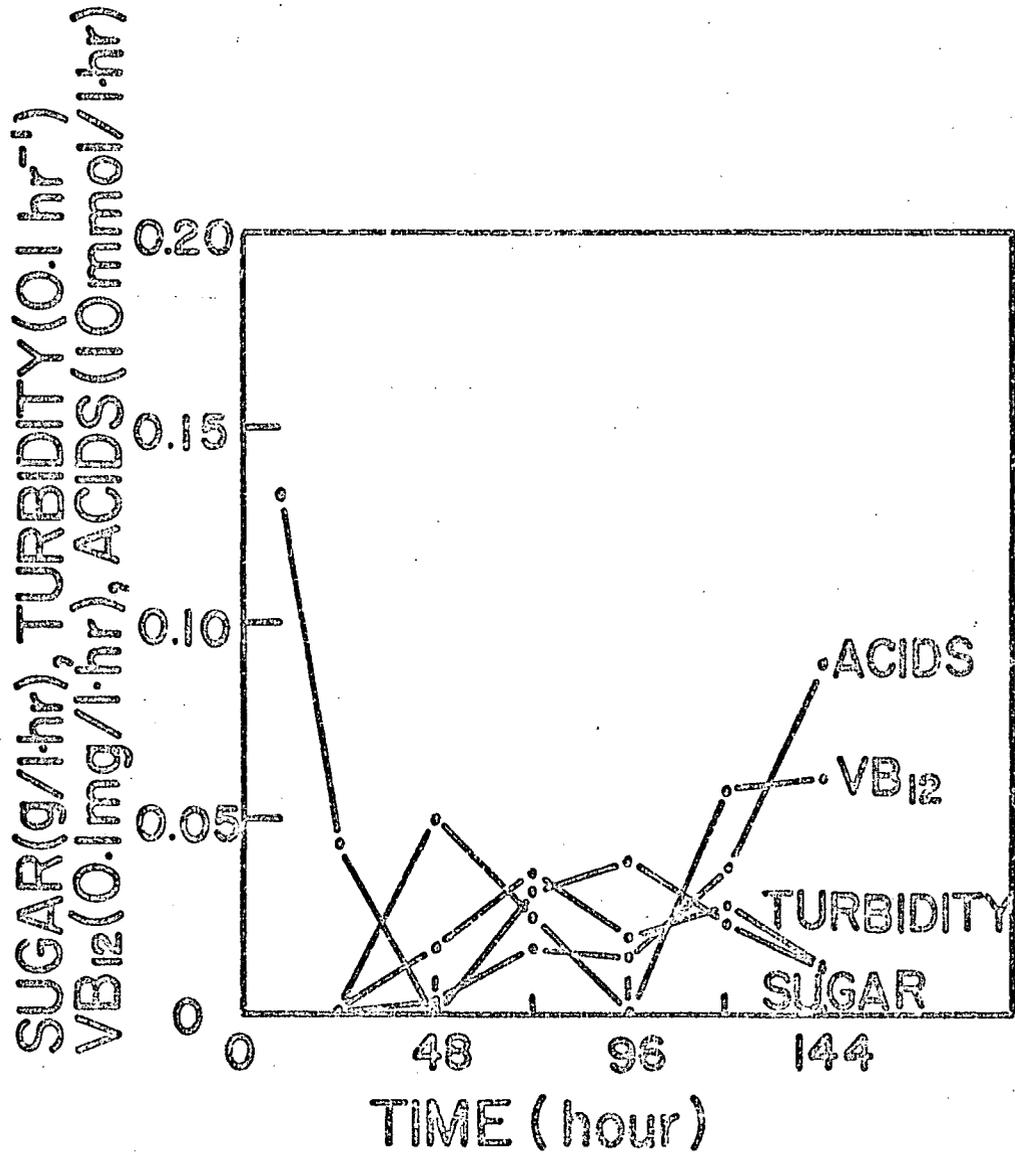500ml BELLCO GLASS
FERMENTOR SSL MEDIUM

Figure 19 FERMENTATION RATE
7L FERMENTOR
SSL MEDIUM

the spent liquor and the manner in which they are consumed is not shown
by a overall sugar analysis.

Riley et.al.(64) noted the aeration was necessary to convert
a precursor of $VB_{12}$ into active $VB_{12}$.  Aso et. al.(33) suggested that
sufficient aeration could be provided through the air liquid interface
at the top of the fermentor if a moderate amount of agitation was supplied.
Our results would tend to confirm those of Aso.  No aeration by bubbling
was used.  However there was opportunity for air to get into the fermentor
during filling, during sampling, or through leakage.  Each fermentation
was agitated and biologically active $VB_{12}$ was produced.

It should be noted that sterilization was important and the
time in the sterilizer should be carefully chosen.  One 7 litre fermen-
tation was contaminated by a black mold which rapidly took over the fer-
mentation from the bacteria.  During sterilization a large amount of
greyish-white, sweet smelling, precipitate was formed in the sulfite
liquor.  This was not observed in the synthetic media and was assumed
to be some wood component.  This precipitate was filtered off before the
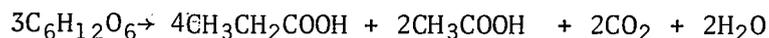medium was inoculated.

The COD of the medium after fermentation is still high although
somewhat lower than that measured before fermentation.  (See Figs. 13, 14,
and 15.)  It should be noted that the COD values after fermentation were
measured with the acetic and propionic acid still present.  In a commercial
operation these would be removed and the COD reduced somewhat more. (114, 115)
With the synthetic medium (Fig. 14) the residual COD is low and may be
attributed to the organic acids present.  However this fermentation appa-
rently does nothing to the lignin type compounds of the spent liquor.

Also it must be remembered that these COD values were measured on treated liquor from which a good deal of pollutants had already been removed.

With certain wastes containing substances which are not consumed by bacteria, COD values may be higher than BOD values. However with wastes which contain only organic, bacterial foods the COD value is in good agreement with the 20 day BOD value. Usually a 5 day BOD is measured as a standard test, this value represents about 60% of the total oxygen demand. In sulfite spent liquor there are many chemicals such as tannins which are not readily oxidizable by bacteria.

Since the COD test is much easier and faster to perform than the BOD test and because it can be applied to a wider variety of materials it was chosen for this work to indicate the change in pollution potential caused by the fermentation.

The ratio of propionic acid to acetic acid was measured and found to be 2.29 for the sulfite liquor medium and 2.05 for the synthetic medium. These results were measured on the 7 litre fermentations. According to some workers (67), the theoretical conversion of sugar to volatile acids is 77%. This is based on the following equation.

$$3C_6H_{12}O_6 \rightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2CO_2 + 2H_2O$$

Following this reasoning conversion ratios of sugar to volatile acids were calculated and are given in Table 12. Some values are higher than 100% especially in the fermentations with low initial sugar content. This suggests that the above equation is wrong or that the Sieber method does not correctly measure the sugar content of the medium before fermentation.

It may be unable to detect sugars in a polymeric form: these could be hydrolyzed during fermentation to produce more monosaccharides which could then be fermented. In any case the equation given above does not apply to pentoses which are present, and which can be used by this bacteria. Also, as the metabolic pathway for any organism is rather complex it would seem that such a simple stoichiometric equation is a rather naive description of the true situation.

According to Pfeifer et. al. (63) the total $VB_{12}$ activity increases rapidly for about 70-120 hours. After this the $VB_{12}$ activity in the medium increases to some extent but the $VB_{12}$ activity inside the cells decreases. In this work the distribution of $VB_{12}$ inside or outside of the cell was not measured as a function of time. Only a terminal result was measured for the 7 litre fermentations which is given in Table 13. To extract the $VB_{12}$ from the medium would require an expensive liquid-liquid extraction stage capable of treating large volumes of liquid. However if the major part of the $VB_{12}$ is inside the cells, the cells can be easily recovered by centrifugation. Then the cells could be broken and the $VB_{12}$ recovered by a much smaller scale liquid-liquid extraction. The $VB_{12}$ in the medium could be recycled to the fermentation to supply a growth factor for the organism. Since the goal of the project was to produce crude $VB_{12}$ suitable for animal feed it would be desirable to have it retained inside the cells for reasons of economy of separation and also the cell bodies, if not toxic, could be utilized by the animal as a protein source.

## 5. Conclusions

The spent liquor from calcium and ammonium base sulfite process pulp mills can be fermented with Propionibacterium freudenreichii, after a portion of the lignin has been precipitated out by pretreatment. The products of the fermentation based on the spent liquor from a one ton per day pulp mill, according to the results found in this study, would be:

0.6 to 1.5 x $10^{-2}$ pounds of total $VB_{12}$ (both in cell bodies and medium)

15 to 20 pounds of acetic acid

30 to 43 pounds of propionic acid

Besides these marketable products there would result a moderate reduction in the COD value of the remaining spent liquor.

The ratio of propionic acid to acetic acid in the product was shown to agree with the literature (67) value of two. However in the case of the spent liquor fermentation the slight increase of this ratio to 2.3 may indicate that a different metabolism is occurring. The conclusion that the fermentation mechanism is different for a synthetic medium containing a single monosaccharide and for sulfite spent liquor is fortified by the shape and position of the volume based rate curves for the fermentation.

For rapid $VB_{12}$ assays, the spectrophotometric method modified for sulfite spent liquor gives good results.

## 6. Recommendations

To reduce the oxygen demand of sulfite spent liquor and produce $VB_{12}$ and volatile acids, the process shown in Fig. 20 is recommended.
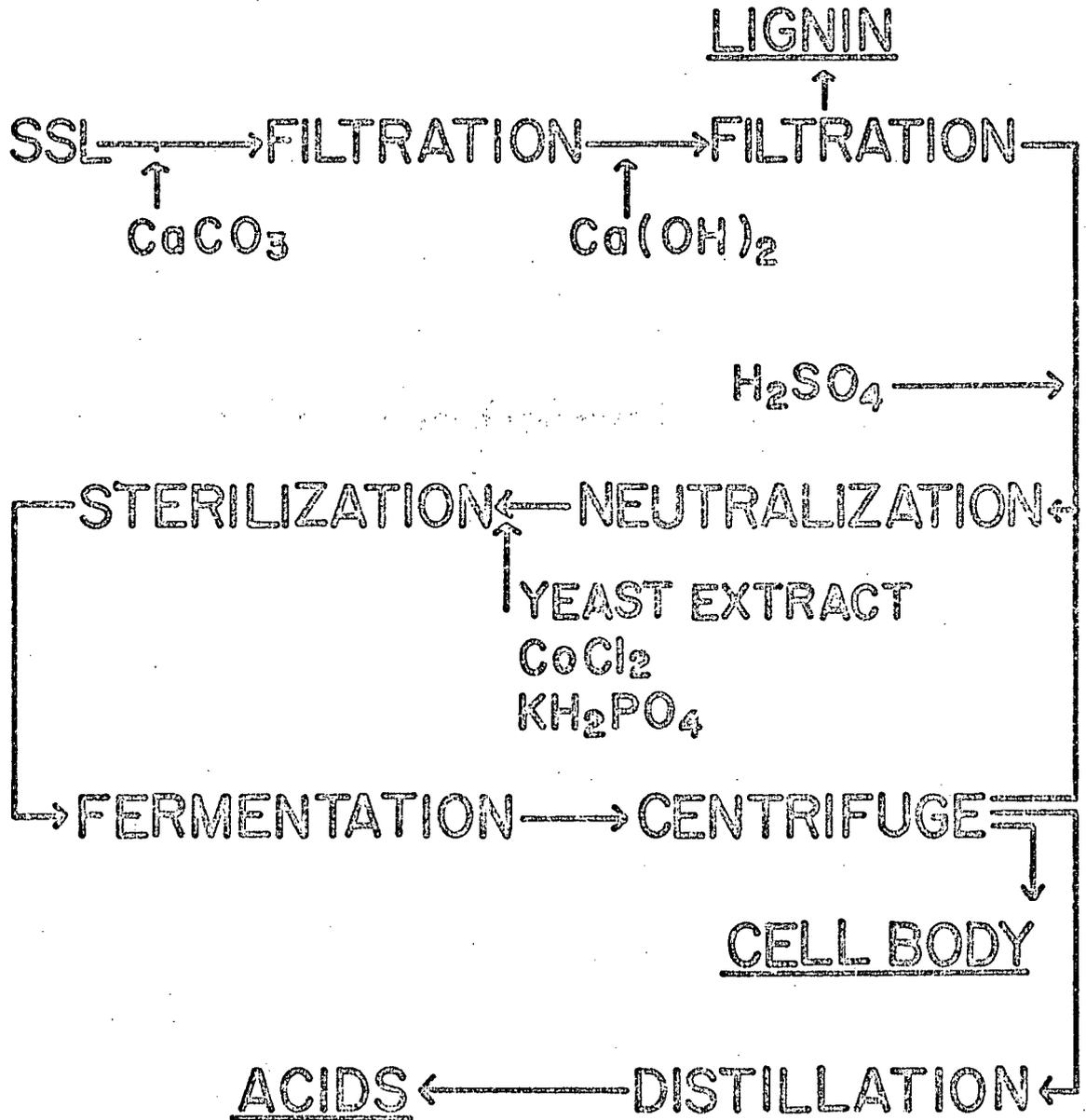
Figure 20  PROPOSED FLOW SHEET

Further work which should be undertaken on the sulfite spent liquor fermentation is:

(a)   Continuous fermentation of 'treated' sulfite spent liquor.

(b)   Fermentation of the lignosulfonate precipitate to give cell bodies or another marketable chemical product.

(c)   The timewise analysis of sugars in the spent liquor media.

(d)   Time study of the distribution of $VB_{12}$ inside and outside the cell.

(e)   Attempt to ferment the non-treated sulfite spent liquor.

## Acknowledgement

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Appendix 1-1  Sugar analysis

Total sugar (reducing substance) in sulfite spent liquor was measured by Rudolf Sieber method (Swedish Standard) (66). The procedure is as follows.

1. Sulfite spent liquor is neutralized with $CaCO_3$ and diluted 10 times with water.

2. 10 ml copper sulfate solution (69.3 g $CuSO_4.5H_2O$ per liter), 10 ml seignette salt solution (350 g $C_4H_4O_6$ KNa.$4H_2O$ and 100 g NaOH per liter), and 20 ml distilled water are heated to boiling in a flask within 3-4 minutes. As soon as the sample has come to the boil, a 10 ml of neutralized and diluted sample is added from a pipette within 15 seconds. Within 60 seconds the sample solution should resume boiling. Exactly 3 minutes after addition of sample, the boiling is discontinued and the flask is abruptly cooled to about 20°C under the cold water tap.

3. While swirling, 10 ml of potassium iodide solution (300 g KI per liter) and then 10 ml of sulfuric acid (250 g $H_2SO_4$ in liter) are added to the flask.

4. This solution is titrated with 0.1N sodium thiosulfate solution and the consumption of 0.1N sodium thiosulfate, expressed in ml, is recorded as "a".

5. Distilled water is substituted in place of the sulfite spent liquor and the above procedure is repeated. The consumption of 0.1N sodium thiosulfate solution at this titration is recorded as "b".

6. Copper sulfate solution is replaced by 10 ml of distilled water. In this case titration is carried out with 0.1N iodine solution. The consumption of iodine solution is recorded as "c".

7. The total consumption "T" of 0.1N sodium thiosulfate which corresponds to the sugar content in 10 ml diluted or 1 ml original sulfite spent liquor is calculated as follows.

$$T = [b - a - c] \text{ ml}$$

8. The conversion of T to reducing sugar is done with reference to the table 77 in "Die Chemisch-Technischen Untersuchungs-Methoden der Zellstoff und Papier Industrie" p. 344 (66).

Appendix 1-2  Volatile acids analysis

To determine the volatile acids 10 ml of sample were acidified with 0.5 - 1 ml of 85% phosphoric acid. This mixture was heated to 130 - 140°C in an oil bath to distill off the volatile acids. After 8-10 ml of distillate were collected, 10 ml of water was added to the content of the distilling flask and the distillation was repeated till 20-25 ml of distillate was collected. The total volume of distillate was titrated with 0.100N NaOH. Calculated on the basis of 1.000N NaOH and of 1000 ml of sample, the volume of caustic consumed gave the number of millimoles of volatile acids.

Appendix 1-3  Ratio of propionic acid to acetic acid (37)

"Using the volatility with steam and based on the presence of propionic and acetic acid only, the ratio of acetic and propionic acid was calculated. 50 ml acidified sample was boiled under reflux for 10 minutes to expell carbon dioxide; replacing the reflux with a condenser, the sample was steam-distilled until 400 ml distillate had been collected; the sum of volatile acids was determined by titrating a portion of the distillate (a); another 200 ml was subjected to half distillation; 100 ml

of distillate was collected and titrated with 0.100N NaOH (b). If

a = total acidity in 200 ml

b = acidity in 100 ml half distillate

then

a = HAC + HPr

b = 0.366 HAc + 0.585 HPr

where HAC and HPr are expressed in terms of ml caustic.

An unsuccessful attempt was made to determine this ratio by a gas chromatographic technique. This would probably be a more convenient and accurate method if proper columns were available."

Appendix 1-4  Lignin measurement

The determination of lignin was carried out as follows (68).

10 cc of the sulfite spent liquor was boiled in a small beaker for 10-15 minutes to expell the free sulfur dioxide. The evaporated water is replaced and 10 cc of a 5% solution of 2-naphthylamine hydrochloride in 3N hydrochloric acid was added while stirring. This mixture was heated on a steam bath for 1 hour. The precipitate formed began to coalesce and soon changed to a soft gummy consistency. On cooling it became hard and brittle. It was filtered on a weighed Gouch filter, completely air dried over night, and finally heated to 100°C. The amount of lignosulfonate was calculated according to the equation

g.Lignosulfonate = g.NLS x 0.793 x 1.22

where NLS is the precipitated naphthylamine ligrosulfonate, 0.793 is a factor for lignosulfonate in NLS, and 1.22 is the factor to correct for the lignin that is not precipitated by the naphthylamine.

Appendix 1-5  Turbidity

Transmittancy (T) is the ratio of the radiant energy trans-
mitted by the sample (P) to the energy incident upon the sample (Po).
Both radiant energies must be obtained at the  same wavelength, with
the same spectral slit width.

$$T = P/Po$$

Transmittancy is usually given in percent.  Since it is seldom possible
to measure these radiant energies directly because of the presence of
a sample cell, it is customary to consider the transmittancy of the sample
as the ratio of the light transmitted by the cell and the sample to the
light transmitted by some arbitrary standard.  In other words, the sample
is compared to a standard.  The transmittancy of this standard is defined
as 100 percent.

Absorbancy, turbidity, or optical density are the negative
logarithm to the base 10 of the transmittancy:

$$- \log T = \log 1/T = \log Po/P$$

where T is expressed as a decimal fraction, not in percent.

Turbidity is a standard to measure the cell growth, and optical
density is used for the absorbancy of the solution.

Appendix 2    COD

This is ASTM D 1252-58T method which is carried out as follows.

1.  A 50 ml sample or an aliquot diluted to 50 ml with distilled
water is placed in a round-bottom flask and 25 ml of 0.25N standard pota-
ssium dichromate solution is added.  The flask is attached to a Friedrichs
condenser and the mixture is refluxed for 2 hours.  It is then cooled

and the condenser is washed down with about 25 ml distilled water.

2. The contents are transferred to a 500 ml conical flask, washing out the reflux flask 4-5 times with distilled water. The mixture is diluted to about 350 ml and after cooling to room temperature the excess dichromate is titrated with 0.25N standard ferrous ammonium sulfate, using ferroin indicator.

3. A blank consisting of 50 ml distilled water instead of the sample, together with the reagents, is refluxed in the same manner.

4. Oxygen demand is calculated according to the following equation.

$$\text{mg/l COD} = \frac{(a-b)c \times 8,000}{\text{ml sample}}$$

where

a = ml $Fe(NH_4)_2(SO_4)_2$ used for blank

b = ml $Fe(NH_4)_2(SO_4)_2$ used for sample

c = normality of $Fe(NH_4)_2(SO_4)_2$

The rate and extent of oxygen depletion is customarily evaluated by the biochemical oxygen demand, BOD, test. This is not a direct measure of organic content, but is a measure of the capacity to consume oxygen using a suitable microorganism. The BOD test has the disadvantage that it requires 5 days for completion.

COD test does not duplicate the BOD test. It may or may not have a consistent ratio to BOD, however there is no fully acceptable substitute for the 5-day BOD test. Both COD and BOD tests measure organic substances, but some of organic compounds are resistant to each of the two tests, and not in the same manner.

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