Eco Process Assistance

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MELISSA

Memorandum of understanding
ETC/GF/MMM/97.012

ESA contract 12922/98/NL/MV

TECHNICAL NOTE 51.1

Improvement of the biodegradation efficiency by hydrogen peroxide and laccase

Version : 1
Issue : 1

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January 2000

DOCUMENT CHANGE LOG

Version	Issue	Date	Observation
1	0	18-02-2000	Draft
1	1	23/05/00	Final version

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1. Introduction

The biodegradation efficiency of faecal material by an inoculum of autochtonous bacteria at thermophilic conditions (55°C) and pH 6.5 was equal to about 30%. It appeared that proteins were biodegraded for about 70% and fibrous material for only 10% (TN41.2). A major part of the non-biodegradable fraction of human faecal material consists of fibrous components. The most recalcitrant components are cellulose, xylan and lignin. Those components are plant material taken up by food and difficult to biodegrade by anaerobic bacteria.
Cellulose is biodegradable by a wide range of organisms, but the biodegradation efficiency is strongly dependent on the structure of the cellulose and the linkage with lignin and hemicellulose. It is known that in anaerobic conditions lignin is hardly biodegraded (TN 41.3).
The combination of two demonstration reactors, the first one operated at pH 6 and fed with faecal material and the second one operated at pH around 7 and fed with MELiSSA cake pretreated with cellulase and xylanase, resulted in a total conversion efficiency of around 53%. For this efficiency calculation the CO_2, CH_4 and volatile fatty acids productions are taken into account. Proteins were converted for 70% and fibres for 44% (TN 43.2).
In this document the improvement of the degradation of faecal material by pretreatment with Fenton's reagent and laccase was investigated.

2. Fenton's reagent

Fenton's reagent (Iron-catalysed hydrogen peroxide) is used to treat a variety of industrial wastes containing a range of toxic organic compounds and complex wastes derived from dyestuffs, pesticides, wood preservatives, plastics additives and rubber chemicals. The process may be applied to wastewaters, sludges or contaminated soils, with the effect being:

- Organic pollutant destruction
- Toxicity reduction
- Biodegradability improvement
- BOD/COD removal
- Odour and colour removal

The following reaction will occur (Bham et al.,1997; Martens et al.,1995):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^oOH$$
$${}^oOH + H_2O_2 \rightarrow HO_2^o + H_2O$$
$$HO_2^o + Fe^{3+} \rightarrow Fe^{2+} + H^+ + O_2$$
$${}^oOH + RH \rightarrow R^o + H_2O_2$$
$$R_i^o + Fe^{3+} \rightarrow Fe^{2+} + product$$
$$2R_j^o \rightarrow product$$
$$R_k^o + Fe^{2+} \rightarrow Fe^{3+} + R_kH$$

The hydroxyl radical is one of the most reactive chemical species known. In the absence of iron, there is no evidence of hydroxyl radical formation when, for example, H_2O_2 is added to a phenolic waste water. As the concentration of iron is increased, phenol removal accelerates until a point is reached where further addition of iron becomes inefficient. Typical range is 1 part Fe per 5-25 parts H_2O_2. The rate of reaction with Fenton's reagent increases with increasing temperature. However, as the temperature increases above 40-50 °C, the efficiency of H_2O_2 utilisation declines. The optimal pH occurs between 3 and 6.

3. Production of hydrogen peroxide

Hydrogen peroxide can be produced by electrolysis of sulfuric acid. At the anode, the following reaction occurs:

$$2HSO_4^- \rightarrow S_2O_8^{2-} + 2H^+ + 2e^-$$

The reaction of this ion with water serves as a commercial preparation of hydrogen peroxide:

$$S_2O_8^{2-}(aq) + 2H_2O \rightarrow H_2O_2(aq) + 2HSO_4^-$$

In the MELiSSA loop, the use of an additional product needs to be avoided. Therefore instead of taking hydrogen peroxide in space, the peroxide can be produced using electrolysis. Following the reaction the sulfuric acid will be recycled. Therefore a beginconcentration of sulfuric acid, water and an electrolytic cell are necessary to produce hydrogen peroxide.

4. Laccase

In the pulp and paper industry traditional bleaching reagents are replaced by enzymes from white rot fungi which are know to naturally degrade lignin. Interests has been particularly focused in the use of one particular type of enzyme, namely laccase.

Laccase is a blue copper enzyme (a polyphenol oxidase) that catalyses the transfer of four electrons from the various organic substrates to reduce dioxygen to water. Laccase can be prepared from cultures of various white rot fungi. The acceptable temperature range runs from 25°C to 60°C with an optimum of ca. 50°C. As with peroxidases, the substrate radicals produced by laccase catalysis undergo various polymerisation, cleavage and other reactions. The redox-potential of laccase is 0.8-1.0 eV. Laccase requires oxygen as a cosubstrate for the oxidation reaction.

Ligninolytic enzymes like laccase attack the lignin directly and hence are more effective. The most important lignin-degrading enzymes are lignin peroxidases, manganese peroxidases and laccase.

5. Set-up of tests

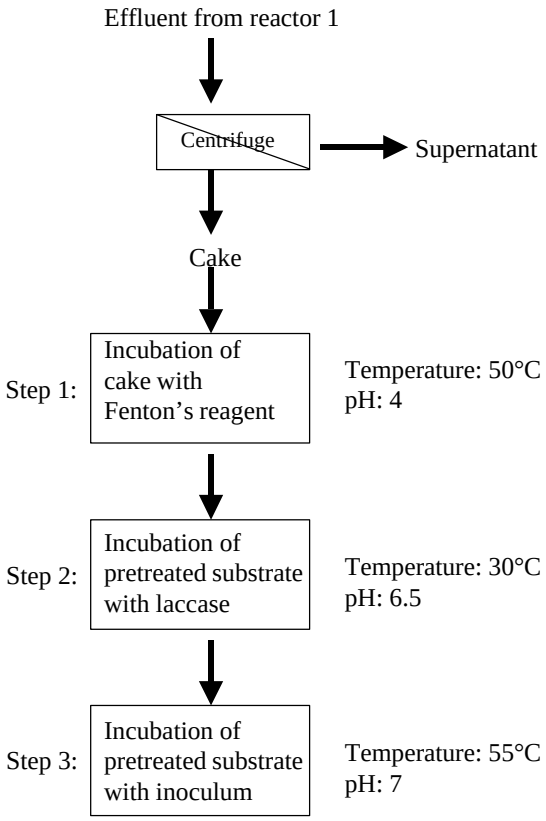

Figure 5-1Schematic overview of the test set-up

5.1 Step 1: Incubation of cake with H_2O_2 and $FeSO_4$ (Fenton's reagent)

100 ml from reactor 1 was centrifuged and the cake was diluted until 100ml with water. This cake, containing the non-biodegraded part of the faecal material, was incubated with H_2O_2 and $FeSO_4$ (Fenton's reagent).

COD and VFA of the cake were measured. The concentration of the H_2O_2 was dependent on the COD of the MELISSA cake. The final COD/H_2O_2 needed to be 1. The final concentration of $FeSO_4$ was 1 g/l.

20 ml of H_2O_2 solution was added to 5 ml $FeSO_4$ solution and 25 ml MELISSA cake. The pH was set at 4 and the test bottles were incubated at a temperature of 50 °C.

The test was set-up in triplicate and lasted for 4 days. Every day the pressure of the test bottles was measured.

At the end of the test COD, DO and pH were measured.

5.2 Step 2: Incubation of substrate / H_2O_2 solution with laccase

The pretreated substrate was incubated with laccase and a buffer. 30 ml of pretreated substrate was incubated with 20 ml laccase solution and 30 ml buffer solution.

The pH was set at 6.5. The test was set-up in quadruple and incubated for 3 days at a temperature of 30°C. Every day the pressure of the test bottles was measured.

At the end of the test the pH and DO were measured.

5.3 Step 3: Incubation of pretreated substrate with inoculum and anaerobic medium

The pretreated substrate was incubated with inoculum from the Melissa reactor 1 (low pH) and an anaerobic medium to provide the inoculum of the necessary elements for surviving and growth. The tests were performed in test bottles of 110 ml. To avoid contamination the tests were prepared under sterile conditions. Test bottles, pipettes, water and needles were autoclaved. Each test was incubated at a temperature of 55°C and the pH was set at 7. The same temperature and pH are used in the MELiSSA demonstration reactor. Several tests were set-up in order to compare the results. The composition of the different tests are shown in Table 5-1.

Table 5-1 Flask content of the different tests expressed as amounts (ml) of different solutions used

Test	*Total volume*	*Inoculum*	*Anaerobic medium*	*Pretreated substrate*	*Starch 1g/l*	*H_2O*
	ml	ml	ml	ml	ml	ml
. Blanc: inoculum $^-$, pretreated substrate$^+$	60	0	20	35	0	5
. Blanc: inoculum $^+$, pretrated substrate $^-$	60	5	20	0	0	35
. Blanc: inoculum$^-$, pretreated substrate$^-$, substrate$^-$	60	0	20	0	0	40
. inoculum $^+$, pretreated substrate$^+$	60	5	20	35	0	0
. inoculum $^+$, starch $^+$	60	5	20	0	35	0

Each test was set-up in quadruple. All the test bottles were flushed with a gas containing 70% N_2 and 30% CO_2 to obtain anaerobic conditions. All test bottles were regularly shaken. The total reported test period was 8 days. During this period pressure in the test bottles was frequently measured and at day 8 COD, VFA, pH and gas composition were analysed.

6. Results

6.1 Step 1: Incubation of cake with H_2O_2 and $FeSO_4$

6.1.1 Before incubation

Characteristics of the cake of reactor 1 (low pH)

Table 6-1 Characteristics of the Melissa cake

Parameters	*Concentration*
COD (mg/l)	15350
VFA (mg/l)	175
DW (g/l)	8.8
ASH (g/l)	1.0
OM (g/l)	7.7

Assuming that 1g OM consists of 0.5 g C, the cake therefore contains 3.9 g/l C =>**0.3 mol/l C**

Taking into account the dilutions, obtained by adding H_2O_2 and $FeSO_4$, the following concentrations were present in the test bottles (Table 6-2).

Table 6-2 Parameters of the Melissa cake taking into account the dilution

Parameters	*Concentration*
COD (mg/l)	7675
VFA (mg/l)	88
OM (g/l)	3.9
C (mmol/l)	160

6.1.2 After incubation

Table 6-3 Parameters of the solution after the incubation with H_2O_2 and $FeSO_4$

Parameters	*Concentration*
pH	3.1
DO (ppm)	7.26
C (mmol/l)	150
COD (mg/l)	5030

The amount of COD removed after the addition of H_2O_2 and $FeSO_4$: **34**%.

The amount of C removed : **8**%.

The COD is the amount of oxygen required for the chemical oxidation of the organic matter. Since the organic matter already contained a lot of oxygen, obtained from the treatment with fenton's reagent, less oxygen was necessary for the COD measurement, therefore the COD result was lower than expected, and a higher COD removal was found.

6.2 Step 2: Incubation of pretreated substrate with laccase

The pH and the DO were measured at the end of the incubation. The results are represented in Table 6-4

Table 6-4 pH and DO after the incubation with laccase

Flask	*pH*	*DO*
1	9.5	2.6
2	9.4	3.0
3	9.3	0.7
4	9.4	0.9

6.3 Step 3: Incubation of pretreated substrate with inoculum and anaerobic medium

6.3.1 Gas production

The evolution of the pressure built-up during the experiment is represented in Figure 6-1 . Both '$Inoculum^+$, $pretreated\ substrate^-$' and '$Inoculum^-$, $pretreated\ substrate^-$' had low pressure readings. The gas found in the test '$Inoculum^-$, $pretreated\ substrate^-$' was of chemical origin. To obtain a chemical equilibrium between gas and liquid phase, CO_2 will evaporate. At the beginning the pressure increased with a high rate in the test '$Inoculum^+$, $starch^+$'. In the test '$Inoculum^+$, $pretreated\ substrate^+$' the pressure increased slowly, but the cumulative gas production at the end of the test for '$Inoculum^+$, $pretreated\ substrate^+$' and '$Inoculum^+$, $starch^+$' evolved to the same value. From the results with the gasanalyser can be concluded that no methane

was found in all 5 tests. This was probably due to the fact that the inoculum was taken from the reactor at low pH. The methanogens are inhibited in this reactor.

The average pH at the end of the test of the different tests are gathered in Table 6-5.

Table 6-5 Final pH of the different tests

Test	*pH*
Blanc: Inoculum $^{-}$, pretreated substrate^{+}	7.14
Blanc: Inoculum $^{+}$, pretrated substrate $^{-}$	6.78
Blanc: Inoculum^{-}, pretreated substrate^{-}	6.71
Inoculum $^{+}$, pretreated substrate^{+}	7.16
Inoculum $^{+}$, starch $^{+}$	6.58

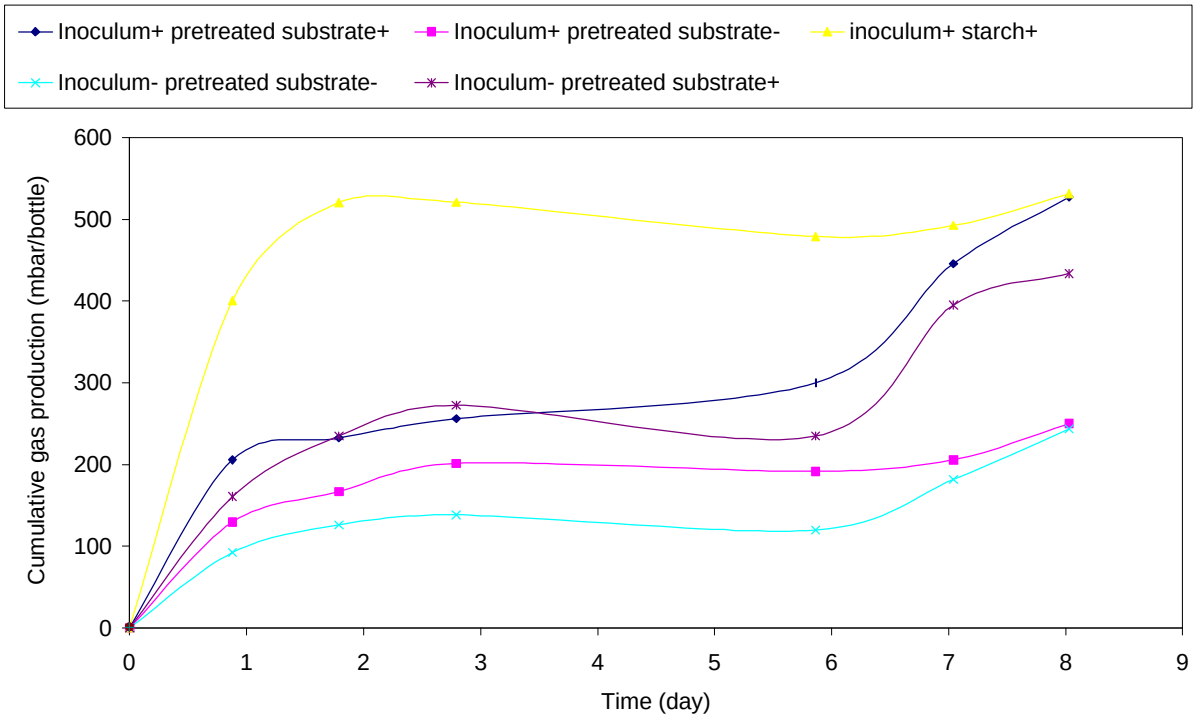

Figure 6-1 Cumulative gas production of the different tests

6.3.2 Volatile fatty acid production

The composition of the volatile fatty acid are represented in Figure 6-2. Volatile fatty acids were found in all different tests. The major part of volatile fatty acids consisted of acetic acid. In the test 'Inoculum^{+}, pretreated substrate^{+}' other VFA were found, namely propionic acid, isobutyric acid, butyric acid and isovaleric acid. These VFA were present only in small amounts.

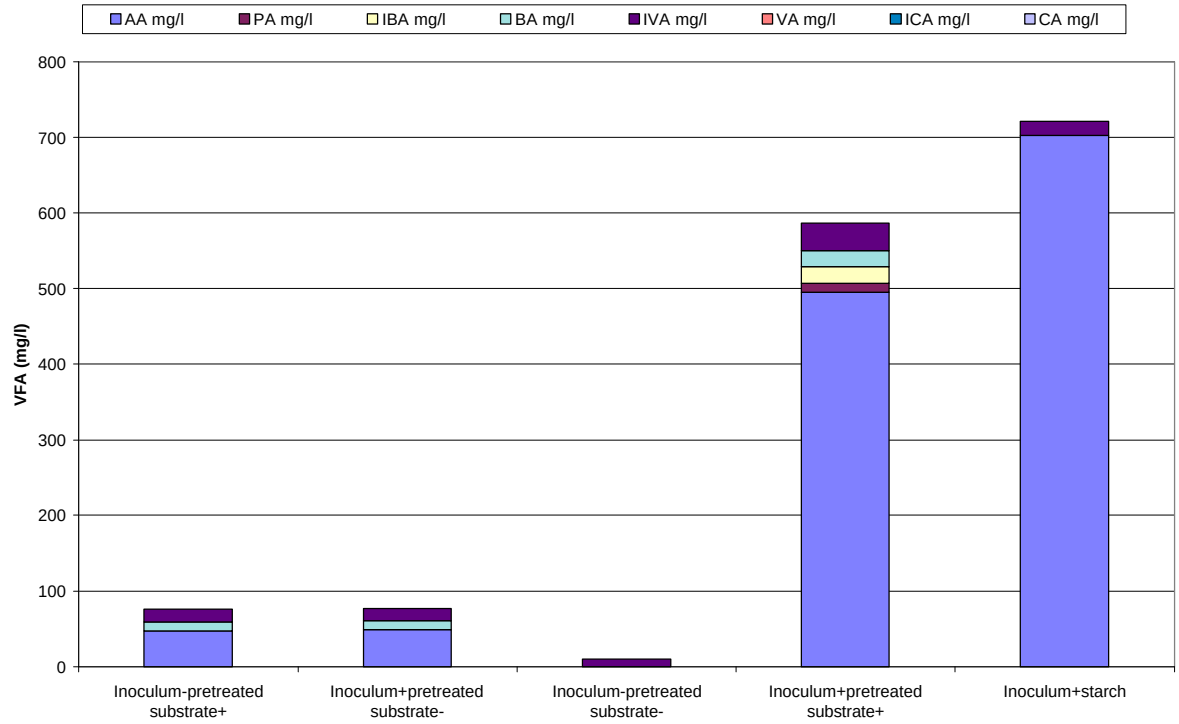

Figure 6-2 VFA composition of the different tests

6.3.3 Mass balance calculations

The biodegradation of organic compounds results in a production of CH_4, CO_2 and volatile fatty acids. The CH_4 production was in these tests nil, since the inoculum was taken from the MELiSSA reactor at low pH, where the methanogens are inhibited. The summation of the CO_2 and VFA production is necessary to have an idea of the amount of biodegraded material. The CO_2 production and the VFA production were transformed into mg/l C.

The total amount of C (VFA and CO_2) found for the test 'Inoculum$^-$ pretreated substrate$^-$' is of chemical origin, and therefore needs to be subtracted from the other tests. The total amount of C found in the test 'Inoculum$^+$, pretreated substrate$^-$' is the biodegradation of the inoculum itself, therefore this results has been subtracted from the test 'inoculum$^+$,starch$^+$' and the test 'Inoculum$^+$, pretreated substrate$^+$'. The total amount of C in the test 'Inoculum$^-$, pretreated substrate$^+$' has been substracted from the test 'Inoculum$^+$, pretreated substrate$^+$'.

Taking into account the degradation of the OM when incubating the cake with H_2O_2 and taking into account the dilutions in the further tests, 0.8 g/l OM was present in the pretreated substrate at the beginning of step3. This amount correspond with 0.4 g/l C. In the test 'Inoculum$^+$, starch$^+$',1 g of OM or 0.5 g C was present. With these data and the results of the test, the conversion efficiencies could be calculated. It can be concluded that 56% of pretreated cake and 79 % of starch was converted (Figure 6-4).

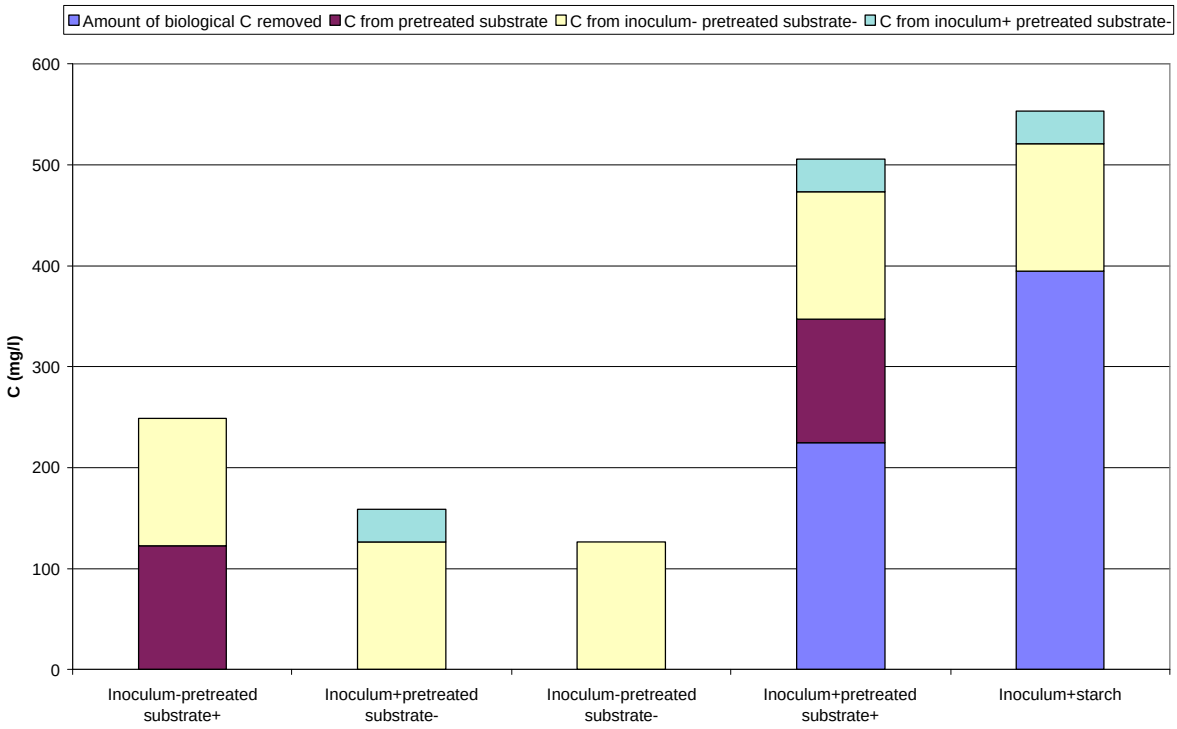

Figure 6-3 VFA and CO_2 production expressed in mg/l C

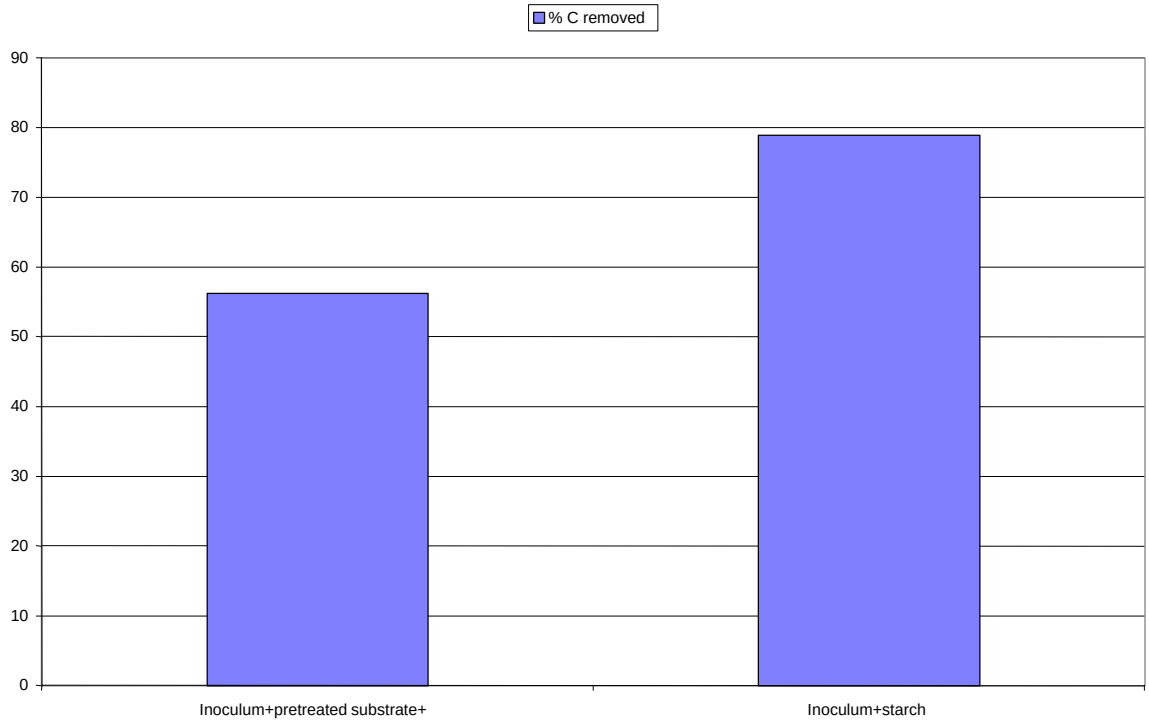

Figure 6-4 Conversion efficiency

6.3.4 Conclusion

The incubation of the cake of the MELiSSA reactor with Fenton's reagent was responsible for a conversion of 8 %. When this pretreated cake was incubated with inoculum and an anaerobic medium a conversion

efficiency of 56 % was found. It can be concluded that the cake can be converted for 60% after the pretreatment with fenton's reagens and laccase and a thermophilic incubation with an inoculum of autochtonous bacteria from faecal material. This in comparison with a 45% biodegradation of the MELiSSA cake without any pretreatment. In Table 6-6 a mass balance calculation was performed to calculate the amount of external products necessary for the pretreatment of 1 g of MELiSSA cake.

Table 6-6 Mass balance calculation of external components

External components	***g/g DW MELiSSA cake***
H_2O_2	0.9
$FeSO_4.7H_2O$	0.07
laccase	0.02

It can be concluded that the pretreatment with Fenton's reagent and laccase is not recommended since a high amount of external products are necessary to increase the conversion efficiency with only 15 %.

7. Pretreatment of cake of reactor 1 and introducing it to reactor 2

7.1 Introduction

Since the degradation of organic material in thermophilic conditions by an inoculum of autochtonous bacteria of faecal material was improved by the addition of fenton's reagent and laccase, a new reactor was started up. This liquefying reactor was operated at pH 8. Cake from reactor 1 was pretreated with fenton's reagent. Dependent on the amount of COD present in the cake, a H_2O_2 solution was prepared in order to have a ratio of $COD/H_2O_2 = 1$. A $FeSO_4$ concentration of 1g/l was used. The cake was pretreated with Fenton's reagent for 4 days at pH 3-3.5 and temperature 50°C. Afterwards a small amount of laccase was added and incubated for 2 days at pH 6.5 and temperature 37°C.

7.2 Problems

7.2.1 Oxygen

When incubating organic material with fenton's reagent a reaction occurs where a lot of oxygen is produced (paragraph 2). This oxygen is both present in the gas phase and in the liquid phase. Before introducing the pretreated cake in the thermophilic anaerobic reactor the oxygen needed to be removed. This could be done by adding laccase to the substrate. Laccase requires oxygen as a cosubstrate for the oxidation of organic material. In the tests with the test bottles, good results were obtained. The dissolved oxygen in the cake could be reduced to an average of 1.8 mg/l. Laccase is scarce and therefore only small amounts were added to the pretreated cake, before introducing it into the liquifying reactor. Probably the amount was not high enough because after two days still a lot of dissolved oxygen was present. Flushing the content with N_2-gas was another option. This method is only useful when small amounts of dissolved oxygen were present.

7.2.2 Sulfate

The $FeSO_4$ in the Fenton's reagent is necessary for the hydroxyl radical formation, when H_2O_2 is added to a waste. After the incubation with Fenton's reagent and introducing the solution into the MELiSSA reactor, the sulfate could be used by the sulfate reducing bacteria present in anaerobic conditions. These bacteria use sulfate as electron acceptor during the oxidation of hydrogen and organic compounds for energy gain. The overall equation for the reduction of sulfate is:

$$2SO_4^{2-} + 8H_2 \leftrightarrow H_2S + HS^- + 5H_2O + 3OH^-$$

Another reactor with sulfate and in the presence of sulfate reducing bacteria is a reaction with acetate:

$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$$

Sulfides can be toxic to bacteria in an anaerobic system at concentrations in excess of 200 mg/l at a pH near neutral (Malina, Pohland, 1992). The sulfate reducing bacteria will compete with the methanogens and in the course of time the methanogens will disappear.

8. Conclusion

The pretreatment of organic material with Fenton's reagent and laccase is responsible for a 8 % C degradation. The Fenton's reagens is mainly responsible for the fragmentation of complex components in more easily biodegradable components. After the pretreatment with Fenton's reagent the cake can be biodegraded for 56% using an autochtonous inoculum of faecal material under thermophilic conditions.

During the incubation of Fenton's reagent with the cake of the MELiSSA reactor a lot of oxygen is produced. Since the thermophilic reactor is anaerobic, the dissolved oxygen needs to be removed before adding the pretreated cake to the MELiSSA reactor. This oxygen can be partly removed by the enzyme laccase, but a lot of enzyme is nessecary. Since the scarceness of this enzyme, it is not realistic. When small amounts of dissolved oxygen are present, the solution can be flushed for at least 10 minutes in order to remove the dissolved oxygen.

The $FeSO_4$ used can be transformed into H_2S by sulfate reducing bacteria. At a cetain contentration H_2S become toxic. The sulfate reducing bacteria will overgrow the methanogens.

Looking at the mass balance calculations it can be concluded that a high amount of external products are necessary for a minor increase in conversion efficiency, therefore it is not recommended to use peroxide and laccase as a pretreatment for the MELiSSA cake. The use of external products can be avoided by using white rot fungi. These fungi are capable to biodegrade lignin. The biodegradation by fungi is at the moment investigated by ATO (Wageningen).

9. References

Bham, A.A. and Chambers, R.P. (1997). Degradation of high molecular weight chlorinated aromatics and aliphatics in bleach plant effluent by Fenton's reagent. Advanced in Environmental Research. 1 (2) 1997.

Malina, J.F. and Pohland, F.G. (1992). Design of anaerobic processes for the treatment of industrial and municipal wastes. Water quality Management Library-Volume 7. p.11, 209.

Martens, D.A. and Frankenberger, W.T. (1995). Enhanced Degradation of Polycyclic Aromatic Hydrocarbons in Soil Treated with an Advanced Oxidative Process- Fenton's Reagent. Journal of Soil Contamination. 4 (2) 1995. p. 1-16.

10. Addendum 1 Composition of anaerobic medium

Anaerobic medium (1 litre)	
Yeast extract	0.4g
Tripticase peptone	0.4g
Resazurine	0.5 ml
0.2 g in 100 ml a.d.	
Solution A in 1 litre a.d.	10 ml
100 g NH_4Cl	
100 g $MgCl_2.2H_2O$	
40 g $CaCl_2.2H_2O$	
Final pH: 4	
Solution B in 1 l a.d.	2 ml
200 g $K_2HPO_4.3H_2O$	
Trace elements in 1 litre a.d.	10 ml
500mg Na2EDTA.2H2O	
150 mg CoCl2.6H2O	
100 mg MnCl2.4H2O	
100 mg FeSO4.7H2O	
100 mg ZnCl2	
40 mg AlCl3.6H2O	
30 mg Na2Wo4.2H2O	
20 mg CuCl2.2H2O	
20 mg NiSO4.6H2O	
10 mg H3BO3	
10 mg NaMoO4.2H2O	
Na_2S (2.5%)	5 ml
18 g/50 ml NaHCO3	16.6 ml
Final pH: 7	