EPAS n.v.
Technologiepark-Zwijnaarde 3
9052 Zwijnaarde
Fax: 32 9221 82 18
Tel: 32 9241 56 18

Laboratory of Microbial Ecology
Faculty of Agricultural & Appl. Biol. Sciences
University of Gent
Coupure L. 653, 9000 Gent
Fax: 32 9264 62 48
Tel: 32 9264 59 76

Melissa
ESA/YCL Contract 130808
Characterisation of I8: Growth on different substrates
TN 20.2
january 1993

ir. L. Meheus

Melissa

TN 20.2

Table of contents

Characterisation of I8: growth on different substrates

1 INTRODUCTION

In the previous technical note (TN 20.1) the identification of a thermophilic proteolytic strain, isolated from a dry anaerobic composting process (DRANCO), was described. The isolated strain proved to be a Thermobacteroides proteolyticus strain. The aim of the experiments was to determine which carbohydrates and proteins could be used by the isolated strain, and what the growth rate on each substrate was.

2 MATERIALS AND METHODS

Media

The liquid medium used for the growth experiments was the basic MS-medium (Meheus, 1993) supplemented with specific proteins and carbohydrates. In order to garantee anaerobic conditions the medium was flushed with N_2.

Analytical techniques

Volatile fatty acids were extracted with diethylether from acidified samples and determined by gas chromatography using a flame ionisation detector coupled to a glass column containing Chromosorb 101.

The NH_{4+}-N-content was determined by steam destillation in a Kjeltec.1002 apparatus under alkaline conditions.

Protein concentrations were measured using a modified dye binding protein assay (Lowry et al., 1951).

The growth of the bacteria was evaluated by measuring the absorbance (610 nm) of the medium. The growth rate was calculated from the slope of the linear part of the growth curve after logarithmic transformation.

Protease activity was asayed with resorufin-labeled casein as substrate. Resorufin-labeled casein is used as an unspecific protease substrate. It is well suited for the detection of trace amounts of protease activities. The determination is done by light absorption measurements (574 nm). One unit of protease activity is defined as the amount of enzyme causing an increase of absorbance (574 nm) of 0.001 per hour.

Description of the experiments

1. Proteolysis

Fifty ml of basic MS medium was supplemented with 3-5 g/l of a specific protein source, repectively bovine serum albumin (BSA), casein, gelatin, bacto-pepton and trypton. Inoculum (5 ml) of the gelatin-grown strain was added. The bottles were incubated at 60 °C and at regular intervals a sample was taken for analyses: determination of the protein content, growth rate (absorbance) and ammonium-nitrogen concentration, protease activity. The final pH was measured.

2. Fermentation of carbohydrates

Fifty ml of basic MS medium was supplemented with 5 g/l of ten different carbohydrates such as starch, cellulose, glucose etc. After inoculation with 5 ml of a culture of the strain, volatile fatty acids and absorbance were determined as a function of time. The final pH was measured.

3 RESULTS

3.1 Determination of the relationship between the light absorbance of a cell culture and the amount of biomass

There exist several methods to estimate the amount of biomass present in a cell culture. One i s the absorbance measurement. Another is the determination of the SS-content (suspended solids). This is done by filtration of the liquid medium over a 0.45 μm filter, and determination of the dry weight of the cells retained on the filter. Cells of T. proteolyticus were grown in basic MS medium supplemented with gelatin.

A full grown culture with an absorbance of 0.52 (610 nm) was related to an SS-value of 130 mg/l. The SS-content of the medium was << 0.0001 mg/l, so there was no interference.

3.2 Fermentation of proteins

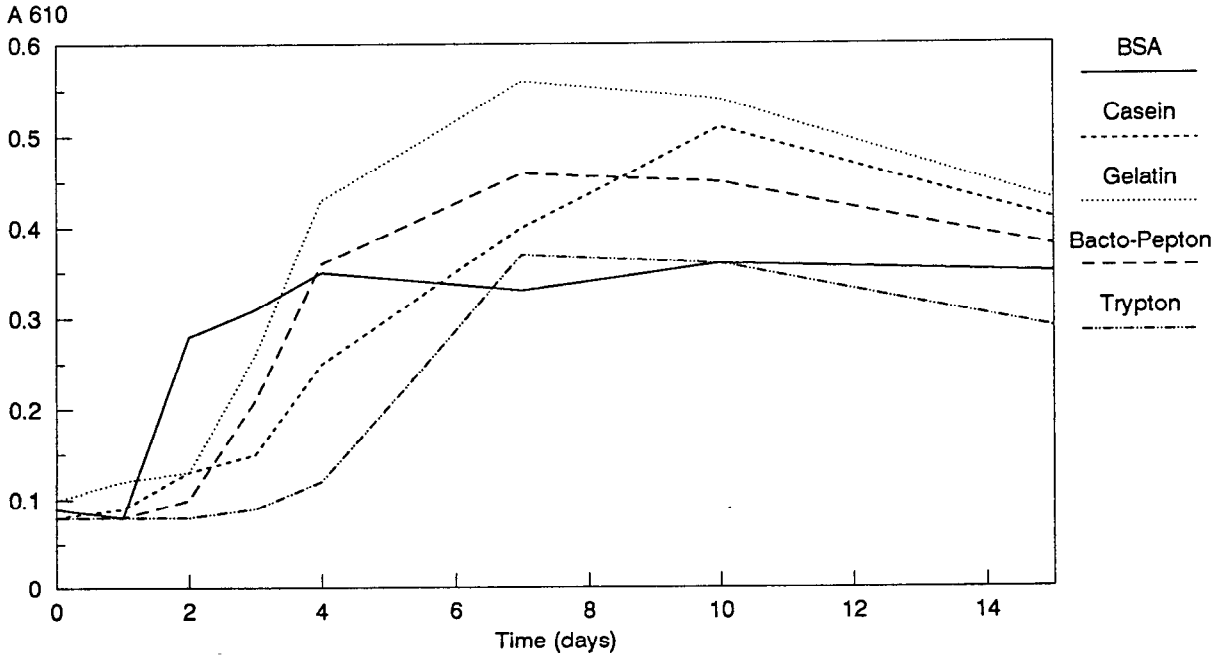
Proteins evaluated in this test were bovine serum albumin, casein, gelatin, bacto-pepton and trypton. The initial cell concentration was about 13 mg SS/l (5 ml of the culture in 3.1 was diluted in 50 ml). Fermentation was monitored during a period of 15 days. In Fig. 1 the growth curves (absorbance measurements) on the different substrates are presented. After converting the figures to log-values, the growth rate was calculated. The growth rates on the different proteins are presented in Table 1. Note that these figures are not μ_{max}-values,but are specific for the circumstances described above.

Table 1. Growth rate of Thermobacteroides proteolyticus on proteïns

	Gelatine	BSA	Caseine	Trypton	Bacto-pepton
Initial protein concentration (g/l)	3.5	3.5	5.1	6.5	5.8
Maximum absorbance (A610 nm)	0.56	0.36	0.51	0.37	0.45
Growth rate (day^{-1})	0.605	0.595	0.343	0.309	0.655

The growth rates on gelatin, BSA and Bacto-pepton were in the same order of magnitude (0.6 day^{-1}). The growth rate on trypton and casein was about half as much. This means that to prevent wash out of the cells in a chemostat a minimum retention time of about 1.6 days (in gelatine-medium) is needed. This has to be verified in a continuous culture study. The maximum absorbance measured for the BSA-culture was low, the initial growth rate was high but growth stopped faster than in the other cultures. The opposite was observed for growth in casein medium; the maximum absorbance measured was about as high as the one in gelatin-medium.

Fig. 1 Fermentation of proteins by *T. proteolyticus I8*
Growth



In Fig. 2 till Fig. 6 protein concentration, volatile fatty acid concentration, ammonium concentration and absorbance are plotted versus time. The net ammonium production, and the efficiency of the protein degradation are presented in Table 2.

Table 2. Breakdown of proteins by Thermobacteroides proteolyticus (t= 15 d).

	Gelatin	BSA	Casein	Trypton	Bacto-pepton
NH_4^+-production (mg/l)	358	176	295	178	298
Degradation (%)	63	48	55	45	65

The curves of the fatty acid production are almost mirror images of the curve of the protein concentrations, which was expected. After the growth had stopped, the fatty acid production stopped but there was still an increase of ammonium. This was probably due to the conversion of indermediate products.

The final concentration of fatty acids in the casein-medium was high. Although the initial growth rate was rather low, growth and fatty acid production went on till day 10. Finally about 55 % of the protein was broken down. The degradation of the other proteins stopped on day 7. The efficiency was lowest for trypton and BSA which corresponded with low maximum absorbance levels and low protease activities (results not shown).

During fermentation there was a slight decrease of the pH which was initially 7. The minimum value was 6.8.

Fig. 2 Fermentation of BSA by *T. proteolyticus I8*

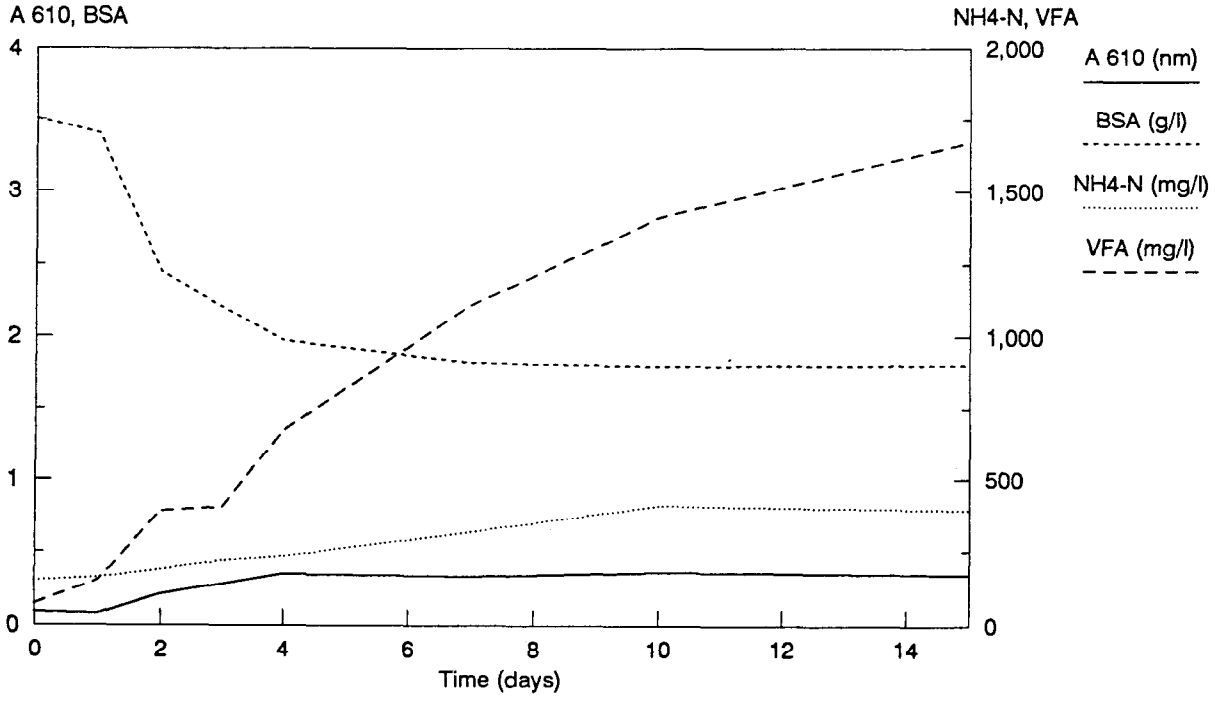


Fig. 3 Fermentation of casein by *T. proteolyticus I8*

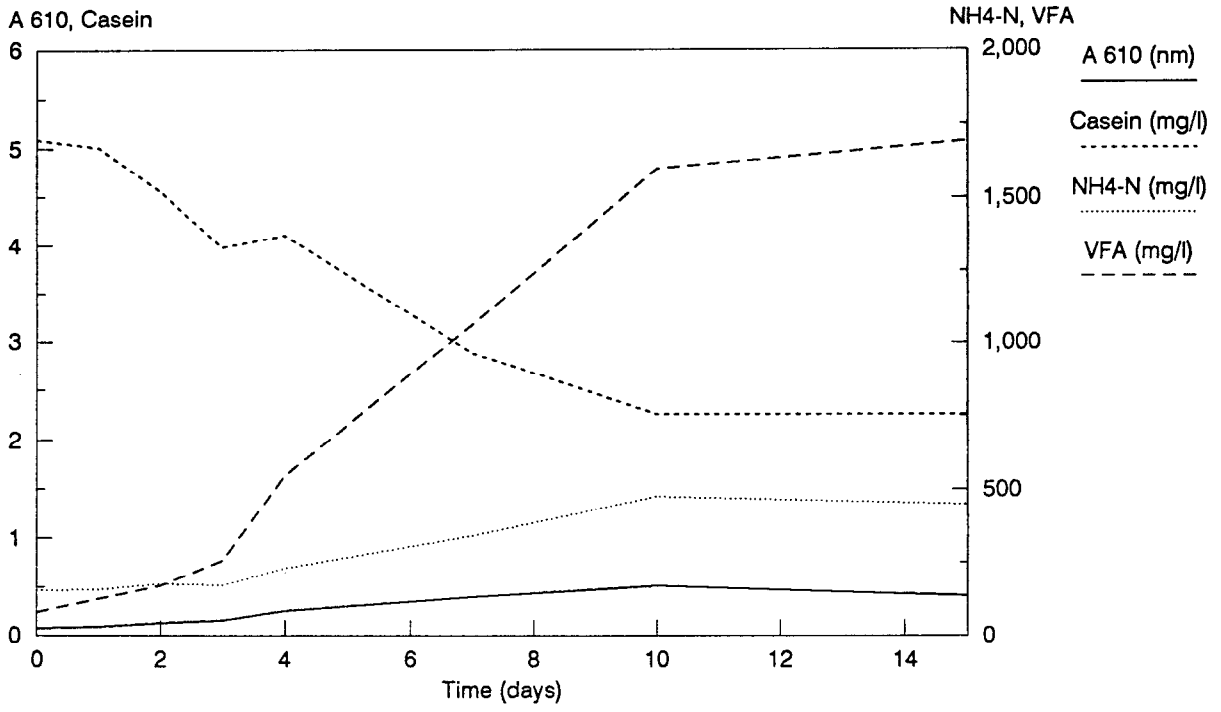


Fig. 4 Fermentation of gelatin by *T. proteolyticus I8*

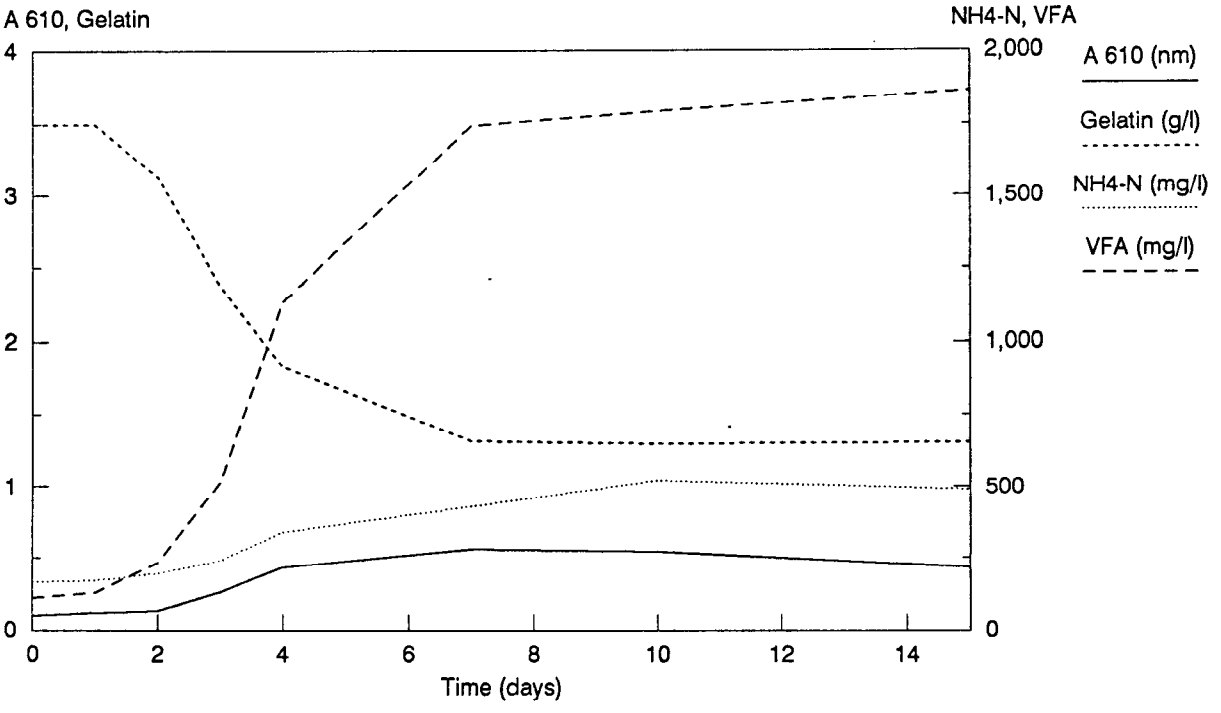


Fig 5 Fermentation of Bacto-pepton by ***T. proteolyticus I8***

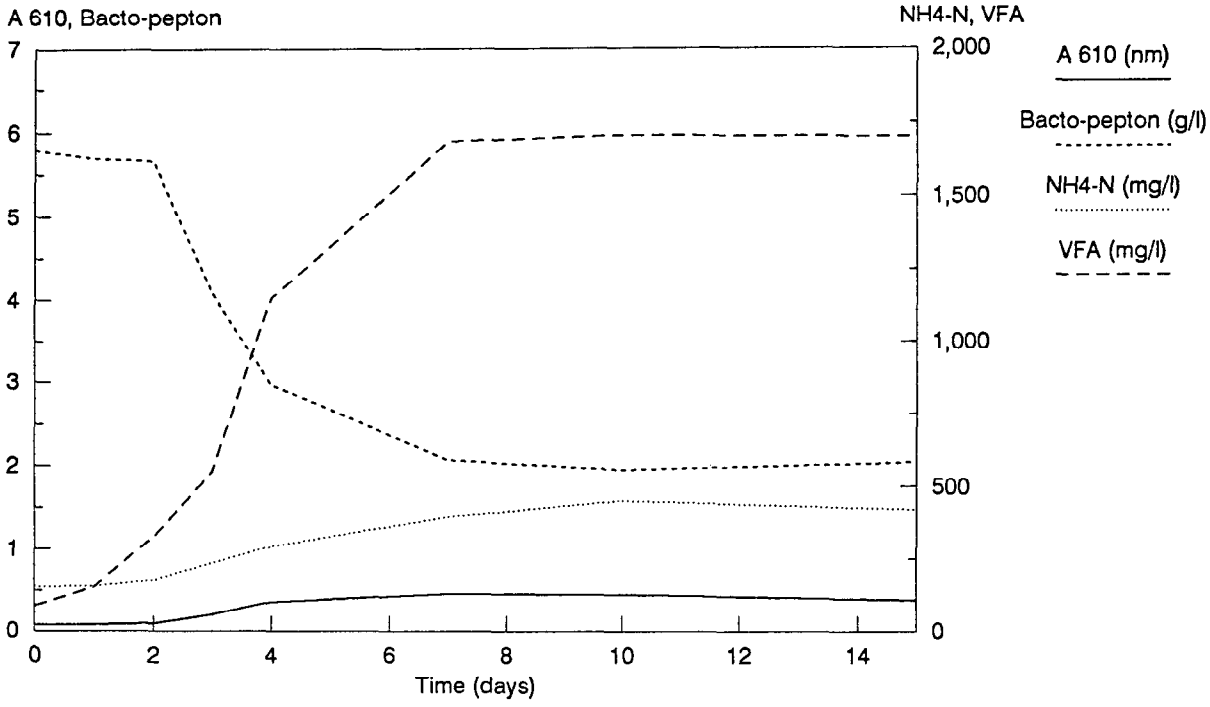
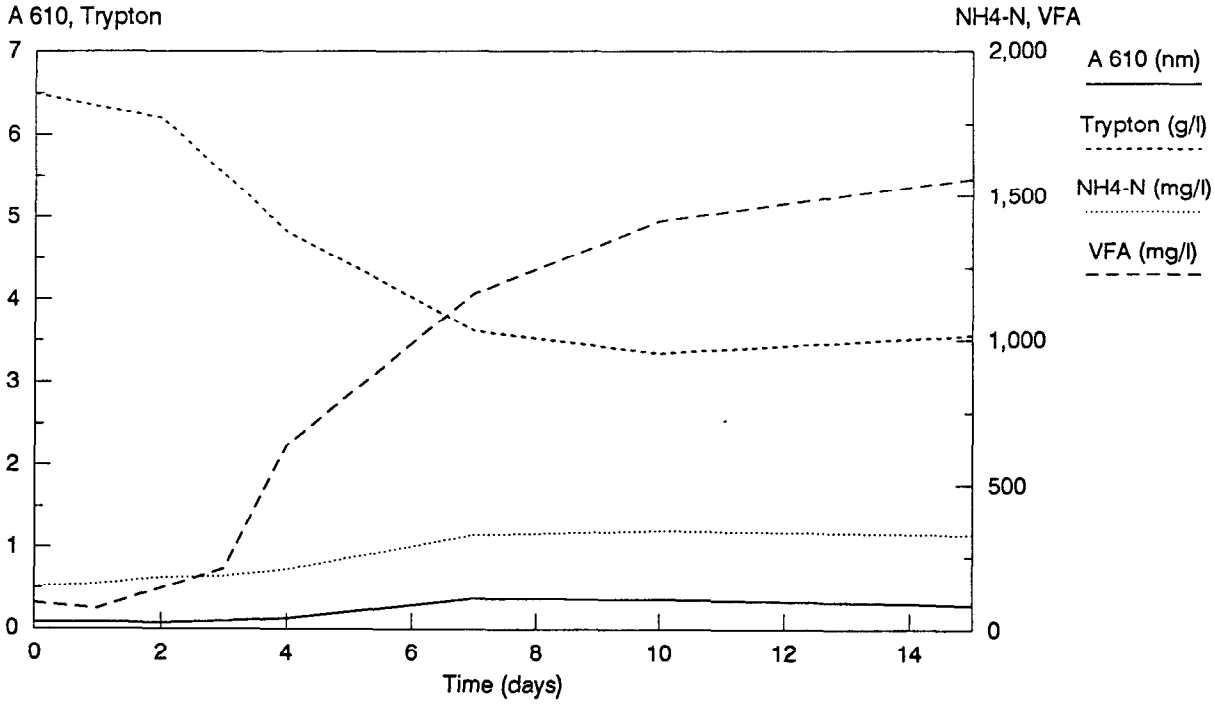


Fig. 6 Fermentation of trypton by ***T. proteolyticus I8***



3.3 Fermentation of carbohydrates

The carbohydrates tested were starch (polymer), raffinose (C_6-C_6-C_6), cellulose (polymer), glucose (C_6), cellobiose (C_6-C_6), maltose (C_6-C_6), sucrose (C_6-C_6), xylose (C_5), fructose (C_6) and mannose (C_6). The degradation and growth were monitored (15 days) by taking samples at regular intervals and analysing them for volatile fatty acids and absorbance. In Fig. 7 and 8 the different growth curves are presented. The observed lag-phase was about one day. This is shorter than the lag-phase observed in the protein degradation test. The growth rates, calculated from the log-transformed measured values, are presented in Table 3. The initial carbohydrate concentration was about 5 g/l. The highest growth rates (1.20 - 1.42 d^{-1}) were observed for glucose, cellobiose, sucrose, fructose and mannose. Lower growth rates (0.71 - 0.93 d^{-1}) were calculated for growth on maltose, xylose, starch, raffinose and cellulose. Growth rates were higher than on protein-substrates.

Growth on maltose went on till day 7 while during fermentation of the other carbohydrates die-off started between between day 3 and 7.

Table 3. Growth rate of Thermobacteroides proteolyticus on carbohydrates

Carbohydrate	Growth rate (d^{-1})	Carbohydrate	Growth rate (d^{-1})
Starch	0.88	Maltose	0.81
Raffinose	0.93	Sucrose	1.26
Cellulose	0.71	Xylose	0.92
Glucose	1.24	Fructose	1.42
Cellobiose	1.20	Mannose	1.28

In Fig. 9 and 10 the volatile fatty acid contents of the media are plotted as a function of time. The low growth rates on starch, cellulose, raffinose, xylose and maltose correspond with a low production rate (slope of the curve) of fatty acids. Remarkable is the high level of fatty acids produced during degradation of maltose (±4000 mg/l). The fatty acid production is almost constant till day 10. This can be related to the prolonged growth phase on this substrate, probably due to the fact to the slow conversion of the molecules to monomers. This slows down (but does not stop) the growth proces. The growth on starch is also slowed down because it has to be converted to glucose molecules.

Fig. 7 Fermentation of carbohydrates by
T. proteolyticus I8
Growth (A)

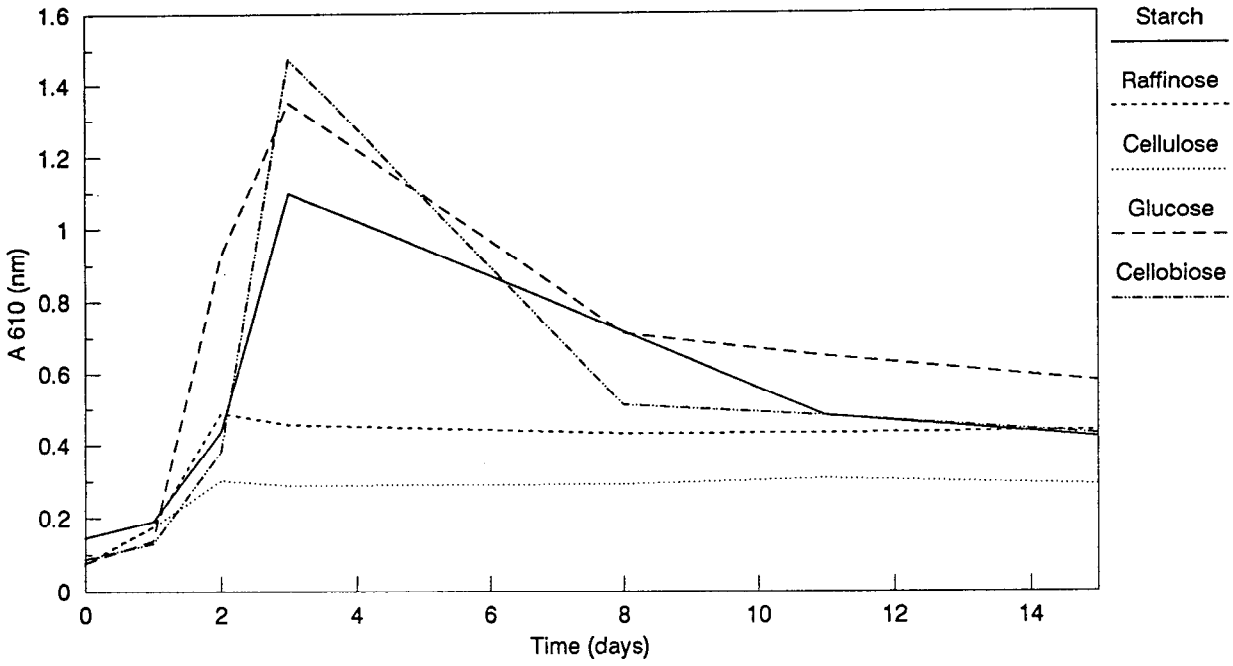


Fig 8 Fermentation of carbohydrates by
T. proteolyticus I8
Growth (B)

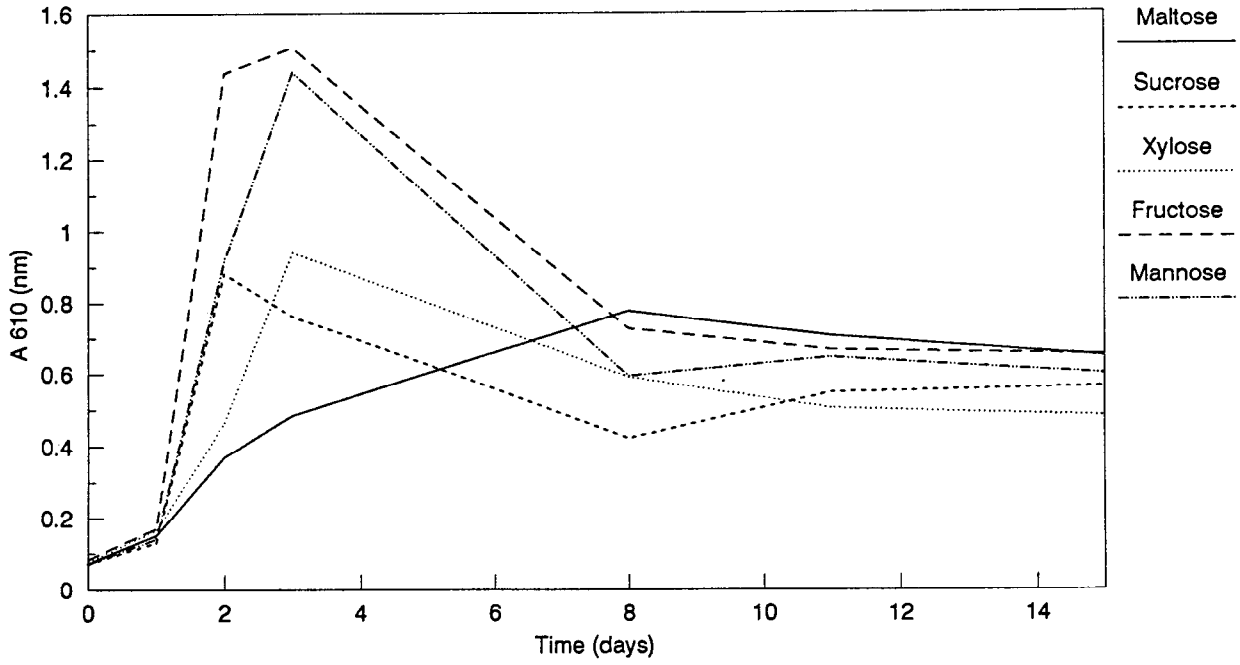


Fig. 9 Fermentation of carbohydrates by
T. proteolyticus I8
Production of VFA (A)

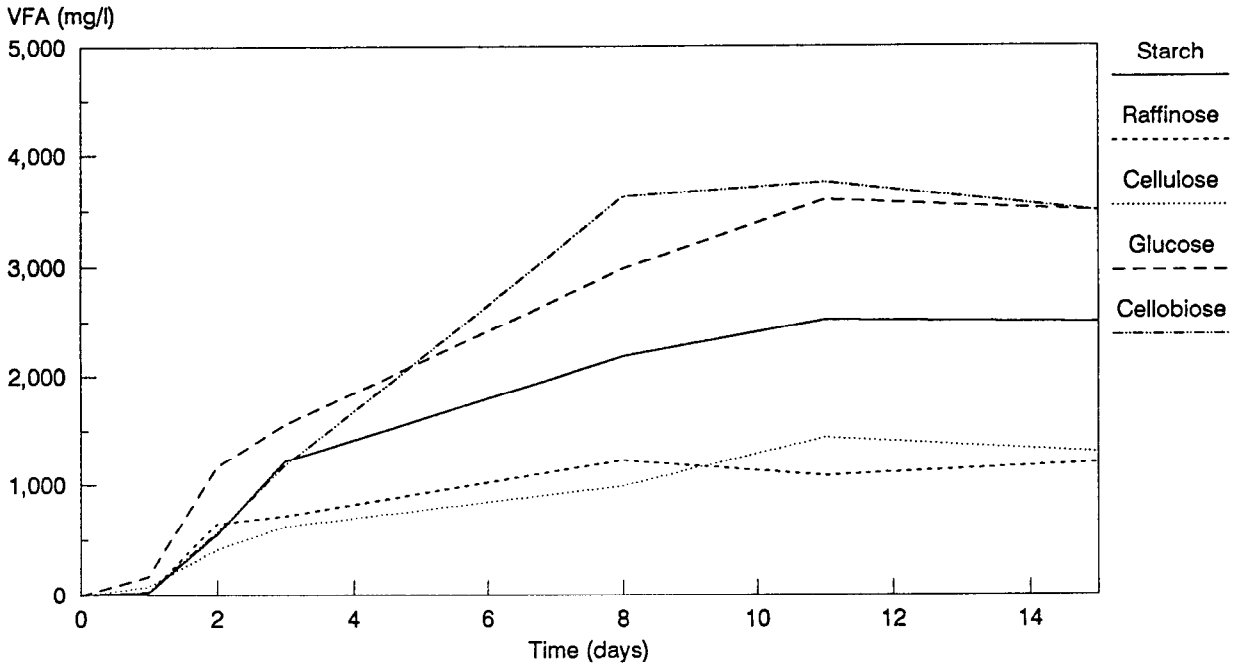
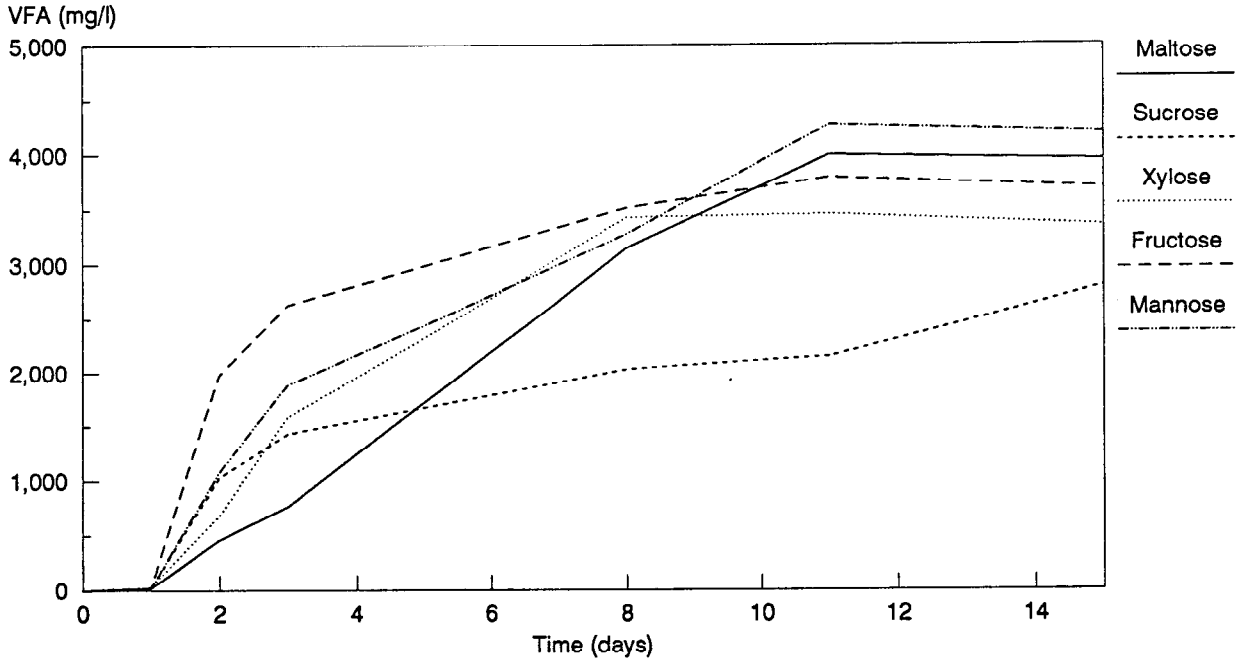


Fig. 10 Fermentation of carbohydrates by
T. proteolyticus I8
Production of VFA (B)



There was a low fatty acid production and a low maximum absorbance during the fermentation of cellulose because the proces of breakdown of the polymer is not fast enough to promote growth. The initial fatty acid production was probably due to the presence of some gelatin (the inoculum was grown in gelatin medium). This was confirmed by the absence growth of the strain on cellulose after several transmissions in fresh medium.
There was almost no breakdown of raffinose. It is a complicated molecule ($C_{18}H_{32}O_{16}$).The breakdown requires special enzymes. The explanation for the initial fatty acid production is the same as for cellulose. But in contrast to cellulose, some growth was observed.

High levels (3000-4000 mg/l) of fatty acids were produced during the fermentation of glucose, cellobiose, maltose, fructose, mannose, xylose and to a lesser extent sucrose. This is an indication for the efficiency of the conversion.

The pH dropped during fermentation (from 7 to 6-6.5).

4 CONCLUSIONS

In Table 4 an overview of the tested substrates is given. A comparison is made concerning growth rate and efficiency of breakdown. The efficiency of breakdown of the carbohydrates was calculated on a COD basis (efficiency is $COD_{VFA}/COD_{initial}$) assuming that the initial COD is 5 g/l (1 g carbohydrate ≈ 1 g COD). There is probably an overestimation because of the presence of some COD in the inoculum.

Table 4 Fermentation of proteins and carbohydrates by Thermobacteroides proteolyticus (summary)

Protein	Efficiency (%)	Growth (day^{-1})	Carbohydrate	Efficiency (%)	Growth (day^{-1})
BSA	48	0.595	Starch	56	0.88
Casein	55	0.343	Raffinose	29	0.93
Gelatin	63	0.605	Cellulose	31	0.71
Bacto-pepton	65	0.655	Glucose	76	1.24
Trypton	45	0.309	Cellobiose	76	1.20
			Maltose	86	0.81
			Sucrose	62	1.26
			Xylose	76	0.92
			Fructose	81	1.42
			Mannose	91	1.28

5 REFERENCES

LOWRY, D. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. J. of Biol. Chem., **193**, 265.

MEHEUS, L. (1993) Melissa TN15.4 ESA/YCL contract 8152/NL/FG.