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MELISSA CCN3

TN 15.2

Table OF CONTENTS

FERMENTATION PATTERN OF *THERMOBACTEROIDES PROTEOLYTICUS*

1. Introduction

An extensive degradation of proteins is necessary in order to obtain an efficient cycling of N, S and C in the Melissa cyclus. This degradation should not form dead end products nor metabolites toxic to the phototrophic bacteria, colonizing the second compartment of the Melissa cyclus. The fermentation of proteins will be examined in order to get a better knowledge of the organic N-metabolism by anaerobic, thermophilic micro-organisms.

The metabolism of *Thermobacteroides proteolyticus* (an anaerobic, thermophilic proteolytic micro-organism) deserves further examination. The degradation of proteins by *T. proteolyticus* will be studied.

In this study, we tested the growth of *T. proteolyticus* on different protein sources. We examined the degradation of proteins and the formation of volatile fatty acids (VFA), ammonium and other end products. The degradation of gelatin by *T. proteolyticus* was studied in relation to the amount of (1) NH_4Cl and (2) yeast extract (YE) supplemented to the medium.

2. Materials and methods

Bacterial strain

Thermobacteroides proteolyticus (ATCC 35245) was isolated by OLLIVIER *et al.* (1985). The thermophilic, anaerobic species was obtained from a methanogenic enrichment inoculated from a thermophilic digestor that was operated on tannery wastes and cattle manure. This organism stains gram negative; it is a nonsporulating bacterium which uses proteins and sugars as substrates (OLLIVIER *et al.*, 1985).

Media

T. proteolyticus (ATCC 35245) was grown anaerobically at 60 °C on the medium described by OLLIVIER *et al.* (1985). Different protein sources, such as peptone, tryptone, casein, cooked meat and gelatin (0,3%) were tested using the above mentioned medium omitting gelatin. Table 1 shows the composition of the medium used for the cultivation of *T. proteolyticus*.

Table 1. Composition of the medium (gelatin as protein source) used for the cultivation of *T. proteolyticus*.

NH_4Cl		1 g/l
$K_2HPO_4.3H_2O$		0,4 g/l
$MgCl_2.6H_2O$		0,1 g/l
L-cystein.HCl (10%)		5 ml/l
yeast-extract (Difco)		1 g/l
resazurin solution (0,2%)		0,5ml/l
mineral solution (BALCH *et al.*, 1979)		25 ml/l
K_2HPO_4	6 g/l	
KH_2PO_4	6 g/l	
$(NH_4)_2SO_4$	6 g/l	
NaCl	12 g/l	
$MgSO_4.7H_2O$	2,6 g/l	
$CaCl_2.2H_2O$	0,16 g/l	
trace mineral solution (BALCH *et al.*, 1979)		5 ml/l
nitrilotriacetic a.	1,5 g/l	
$MgSO_4.7H_2O$	3 g/l	
$MnSO_4.2H_2O$	0,5 g/l	
NaCl	1 g/l	
$FeSO_4.7H_2O$	0,1 g/l	
$CoSO_4$	0,1 g/l	
$CaCl_2.2H_2O$	0,1 g/l	
$ZnSO_4$	0,1 g/l	
$CuSO_4.5H_2O$	0,01 g/l	
$AlK(SO4)_2$	0,01 g/l	
H_3BO_3	0,01 g/l	
$Na_2MoO_4.2H_2O$	0,01 g/l	
gelatin (10%)		30 ml/l
Na_2S (1%)		20 ml/l
$NaHCO_3$ (10%)		5 ml/l
final pH: 7,0		

Analytical methods

Growth was measured by light scattering at 610 nm.

The composition of the gas in the headspace of the tubes was analysed with a gas chromatograph (Intersmat 122 M). Carbon dioxide was separated with a Porapak column while H_2 and N_2 was separated with a carbosieve column.

Fermentation products (such as acetic acid, isobutyric acid, etc.) formed during growth were routinely determined by gas chromatography, using a flame ionization detector.

Protein concentrations were determined using the Bradford dye-binding protein assay (BRADFORD, 1976).

The **NH_4-N content** of the medium was determined by steam distillation in a Kjeltec.1002 apparatus under alkaline conditions. **Kjeldahl-N** was determined similarly, after conversion to NH_3 by digestion.

The protease activity was assayed with resorufin-labeled casein as substrate. Resorufin-labeled casein is used as an unspecific protease substrate. It is especially well-suited for the detection of traces of protease activities. The basic assay procedure involved incubation of samples in a final volume of 200 µl containing 50 µl resorufin-labeled casein (0,4% w/v) as substrate. Incubations were at 60 °C in sealed tubes for 30 minutes and were terminated by the addition of 80 µl 5% (w/v) trichloroacetic acid (TCA). The samples were centrifuged and the absorbance of the supernatans was determined at 574 nm. Control incubations were those in which water was added instead of the sample solution.

Definition of 1 unit protease activity:
One unit of protease activity is defined as the amount of enzyme causing an increase in A_{574} of 0,001 per hour.

3. Results

3.1. Fermentation of different proteins by *T. proteolyticus*

Table 2 compares growth in relation to the specific protein source used for the cultivation of *T. proteolyticus*. The N-balance before and after growth of *T. proteolyticus*, in media containing different protein sources is also represented in Table 2. The production of VFA by the organism in media with different protein sources is shown in Fig. 1

Table 2. Comparison of fermentation pattern and N-balance, before and after growth of *T. proteolyticus* (ATCC 35245) in media containing different protein sources.

	peptone	tryptone	casein	cooked meat	gelatin
Growth A 610 nm	0,158	0,098	-	-	0,128
Kj-N (mg/l)	797	749	747	663	775
NH_4-N start (mg/l)	255	260	255	253	252
NH_4-N end (mg/l)	408	367	399	384	419
N_p (%) degraded	66	72	55	46,5	68
Org-N (%) hydrolyzed	28	22	29	32	32
Final pH	7,2	7,6	7,2	7,2	7,3
acetic acid (mg/l)	961	84	153	0	209
isobutyric acid (mg/l)	99	49	0	74	41
isovaleric acid (mg/l)	280	210	77	875	133
propionic acid (mg/l)	0	0	36	0	0

*N_p: nitrogen from not degraded proteins

Experimental conditions: Products were measured after 29 days growth in rubber bung sealed flasks that contained 200 ml medium with 0,3% substrate. The inoculum of *T. proteolyticus* was grown on gelatin. All flasks were incubated at 60 °C under stationary conditions.

T. proteolyticus grew well on a medium containing peptone and gelatin as a protein source. It was not possible to measure growth of the organism in media containing casein and cooked meat, because these proteins cause initial turbidity when they are soluted in water.

The hydrolysis of different proteins by *T. proteolyticus* ranged from 47% -71%. The amount of organic-N hydrolysed during the fermentation of different proteins fluctuated between 22% -32% (Table 2). Gelatin is degraded by *T. proteolyticus* for 69%, but only 32% of the initial organic-N is mineralized to NH_4-N.

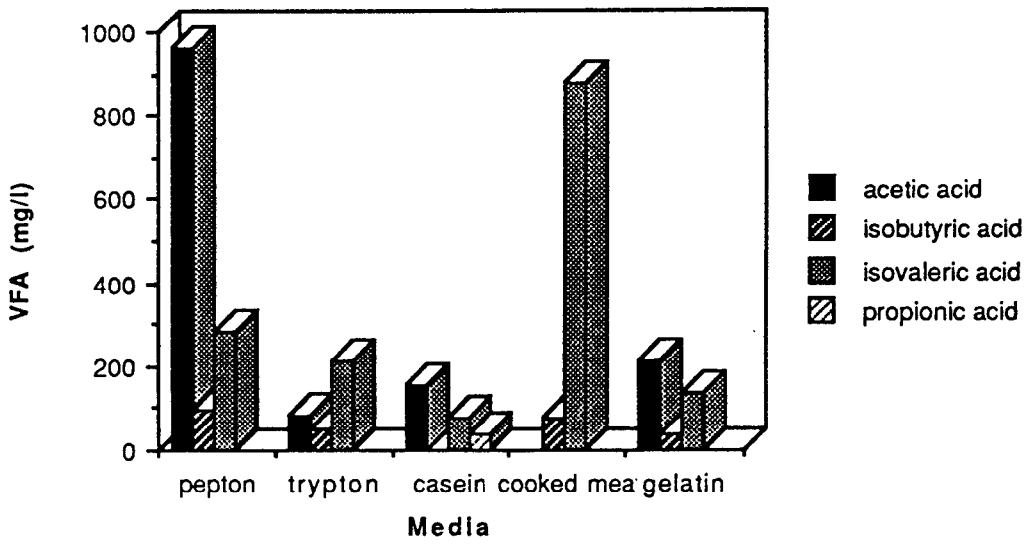

Figure 1. Production of VFA by *T. proteolyticus* in media with different protein sources.

The major products formed during all the protein fermentations were acetic acid, isovaleric acid and ammonia, with CO_2 and H_2 produced as gasses (except when cooked meat was used as a protein source). Traces of isobutyric acid and propionic acid were also formed (Fig. 1). The highest amount of acetic acid (960,6 mg/l) was produced on a medium containing peptone as a

protein source and major quantities of isovaleric acid (875,4 mg/l) were formed on the medium containing cooked meat.

Gelatin is well soluble in water, easily to handle and therefore choosen as the protein substrate for studying more in detail the degradation of a protein by *T. proteolyticus*.

3.2. Fermentation of gelatin by *T. proteolyticus*

The organism was grown under N_2 gas phase in aluminium crimp sealed flasks containing 200 ml medium, with 0,5% gelatin. The time course for gelatin degradation to fermentation end products is shown in Fig. 2.

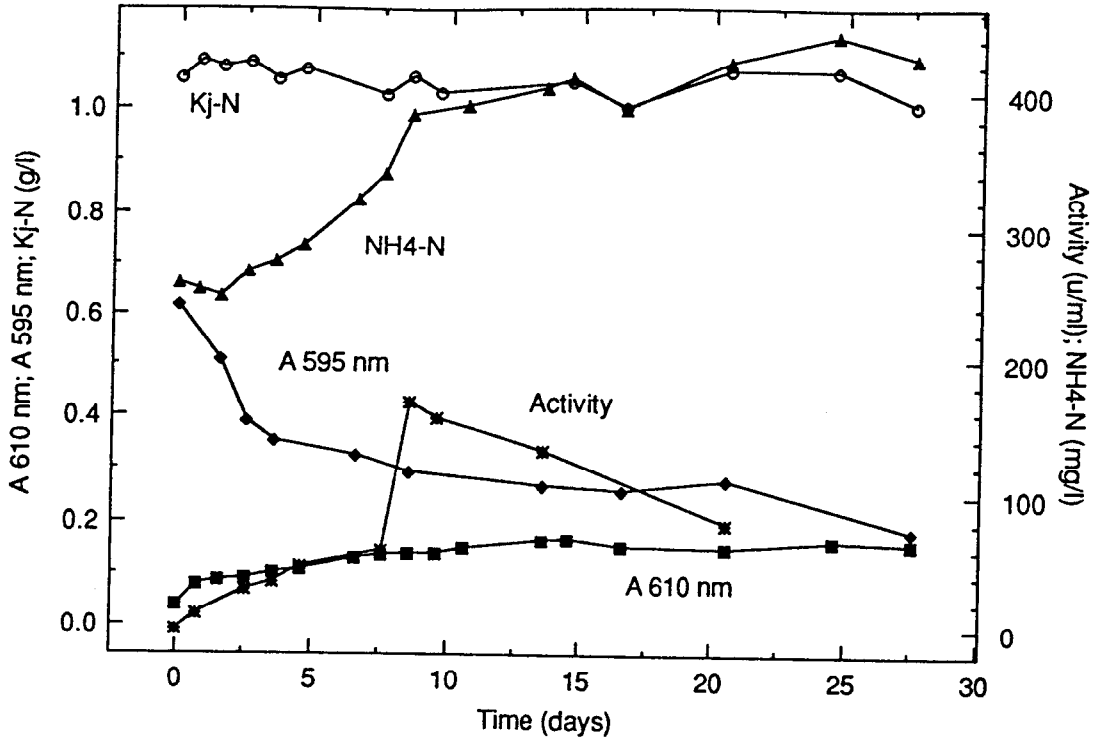

Figure 2. Fermentation time course of *T. proteolyticus* on gelatin.

When Bradford reagent was added to the sample, the gelatin concentration could be determined by measuring the absorbance at 595 nm. The protein substrate was consumed during growth, as shown by a decrease in absorbance at 595 nm. Gelatin was not

metabolised completely: 76% of the initial protein concentration was degraded but only 26% of the organic-N was completely mineralized towards NH_4-N. It is likely that *T. proteolyticus* is unable to utilize part of the substrate. The degradation products from gelatin, formed during the fermentation process are shown in Table 3.

Table 3. Growth and end product formation by *T. proteolyticus* after 32 days incubation at 60 °C.

Growth (A 610 nm)	0,157
A 595 nm	0,381
Final pH	7,11
NH_4-N start (mg/l)	258,6
NH_4-N end (mg/l)	455,4
Org-N start (mg/l)	799,4
Org-N end (mg/l)	587
Kj-N (mg/l)	1042
Np degraded (%)	76
Org-N degraded (%)	28
acetic acid (mg/l)	512,8
isobutyric acid (mg/l)	34,6
isovaleric acid (mg/l)	101,3

Acetic acid (513 mg/l) was the main acid produced, with H_2 and CO_2 produced as gasses. Isovaleric acid and isobutyric acid were produced as trace products (< 1 mM).

A sample was filtered through a millipore filter (0,45 µm) and the Kj-N in the filtrate was determined. As such, a N-balance could be set up:

$$Kj\text{-}N = NH_4\text{-}N + Nb + Np + Nrest$$

with Nb: biomass-N
Np: nitrogen from not degraded proteins
Nrest: non-protein-N; non-biomass-N

t = 0 d 1058 mg/l = (258,6 + 0 + 799,4 + 0) mg/l

t = 32 d 1042 mg/l = (455 + 16 + 185 + Nrest) mg/l

Nrest = 386 mg/l

Hence, ca. 28% of the initial organic-N is converted.

3.3. Fermentation of gelatin by *T. proteolyticus* in function of different NH_4Cl concentrations

The organism was grown in flasks containing 200 ml medium, with 0,3% gelatin and different NH_4Cl concentrations. The time course for gelatin degradation in function of the NH_4Cl concentration is presented in Fig. 3. The growth of *T. proteolyticus* in media with different concentrations of NH_4Cl was compared and is shown in Fig. 4 and the NH_4-N production in the different media is demonstrated in Fig. 5.

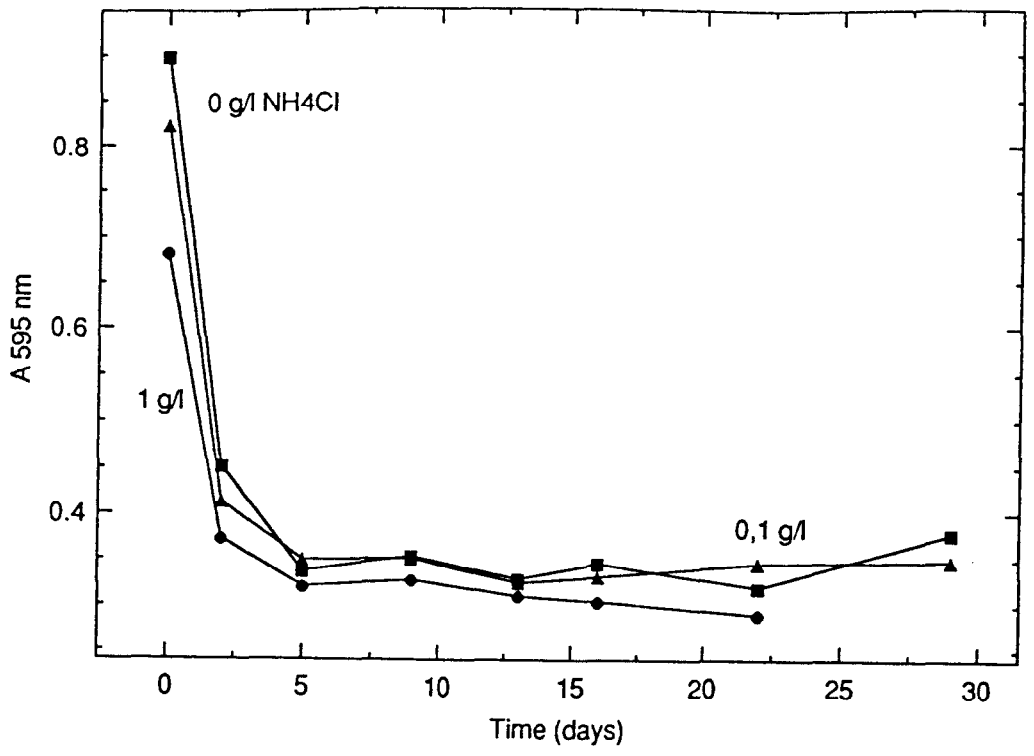

Figure 3. Gelatin degradation by *T. proteolyticus* in function of different NH_4Cl concentrations.

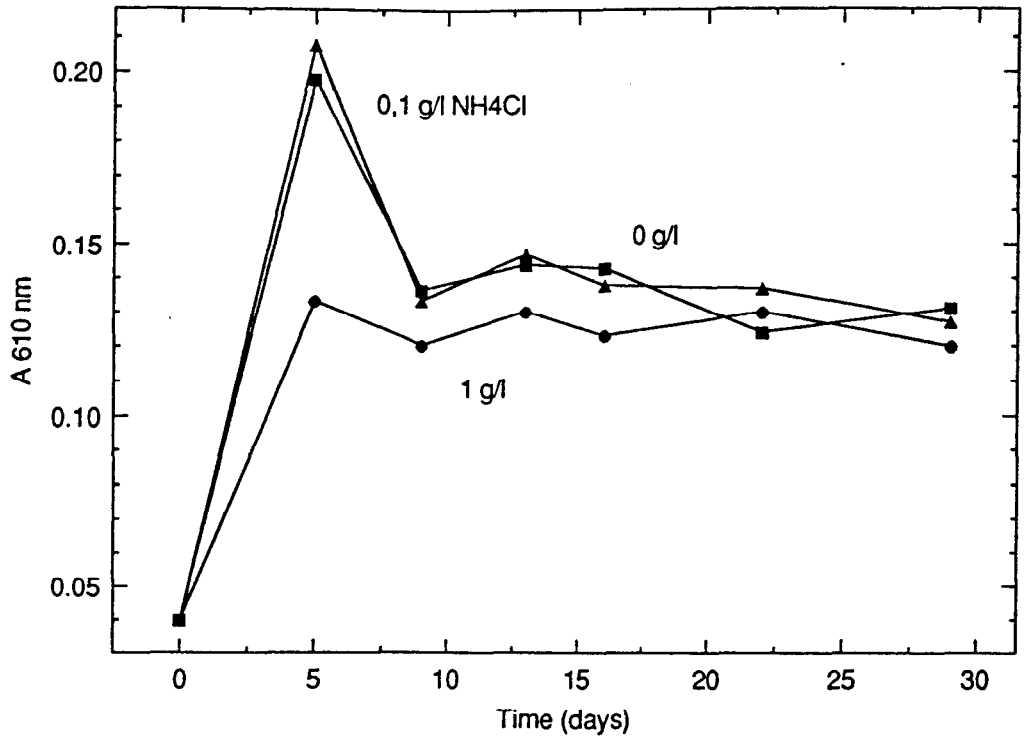

Figure 4. Growth of *T. proteolyticus* in media containing different concentrations of NH_4Cl.

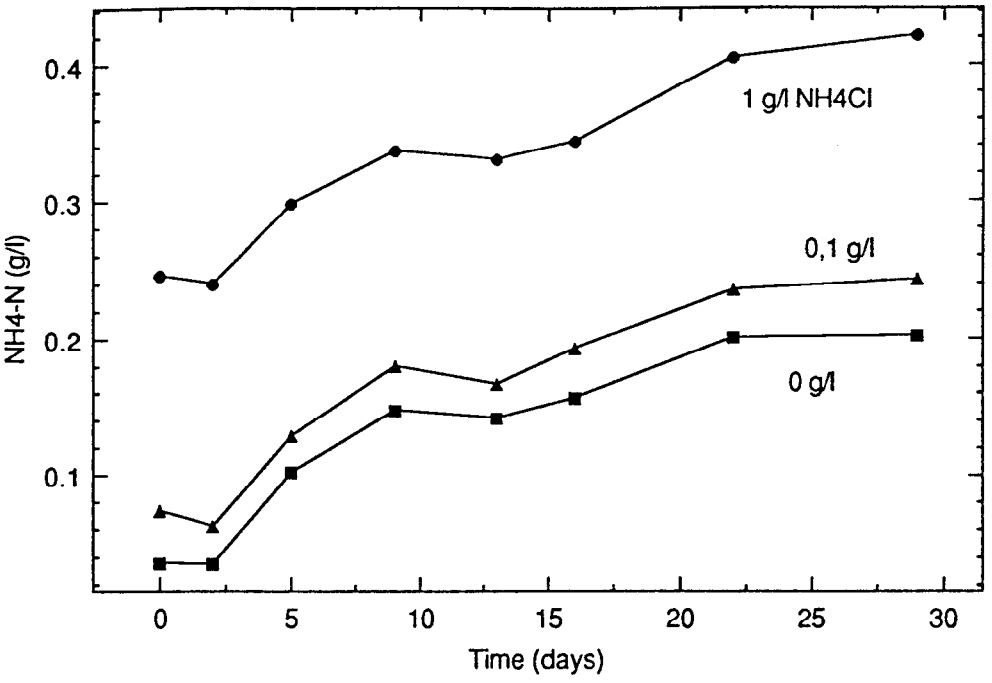

Figure 5. NH_4-N production by *T. proteolyticus* in media containing different concentrations of NH_4Cl.

Table 4 compares (1) growth and (2) gelatin degradation by the organism in relation to different amounts of NH_4Cl added to the medium. The N-balance before and after growth of *T. proteolyticus* is also represented in Table 4. The production of VFA in media with different NH_4Cl concentrations is shown in Fig. 6.

Table 4. Comparison of fermentation pattern and N-balance, before and after growth of *T. proteolyticus*, in media containing different NH_4Cl concentrations.

NH_4Cl (g/l)	0	0,1	1
Growth (A 610 nm)	0,132	0,146	0,124
Kj-N (mg/l)	512	585	794
NH_4-N start (mg/l)	36	74	246
NH_4-N end (mg/l)	206	257	443
N_p (%) degraded	77	78	81
Organic-N (%) hydrolyzed	35,5	36	36
Final pH	7,6	7,4	7,4
ΔA 595 nm	0,639	0,543	0,454
acetic acid (mg/l)	461	374	818
butyric acid (mg/l)	16	36	0
isobutyric acid (mg/l)	42	45	55
valeric acid (mg/l)	13	0	0
isovaleric acid (mg/l)	121	115	131
capronic acid (mg/l)	22	0	0
propionic acid (mg/l)	0	40	0

Experimental conditions: Products were measured after 35 days growth in rubber bung sealed flasks that contained 200 ml medium with 0,3% substrate. All flasks were incubated at 60 °C under stationary conditions.

By adding different amounts of NH_4Cl (0 - 1 g/l) to the medium, growth of *T. proteolyticus* was not influenced. Gelatin was degraded with the same velocity in the three different media. Almost 78% of the protein content is hydrolysed, but only 36% of the organic-N is completely mineralized to NH_4-N. As such, there

are a lot of nitrogenous endproducts of unknown origin (amino acids, amines etc.) which are left as organic-N.

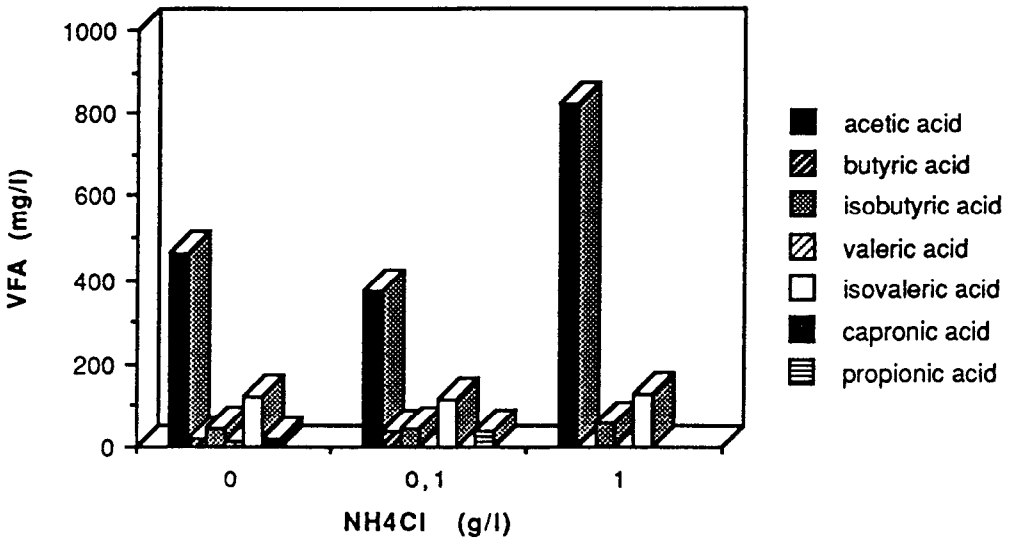

Figure 6. Production of VFA by *T. proteolyticus* in media with different NH_4Cl concentrations.

More acetic acid, isobutyric acid and isovaleric acid were formed in the medium containing 1 g/l NH_4Cl (Fig. 6).

Conclusions

By adding different amounts of NH_4Cl to the medium, following results were obtained:

(1) growth of *T. proteolyticus* was not influenced

(2) gelatin was degraded with the same velocity

As such, ammoniumchloride can be omitted.

3.4. Fermentation of gelatin by *T. proteolyticus* in function of different yeast extract concentrations

The organism was grown in flasks containing 200 ml medium, with 0,3% gelatin and different yeast extract concentrations. The time course for gelatin degradation in function of the YE concentration is shown in Fig. 7. The growth of *T. proteolyticus* in media with different concentrations of YE was compared and is presented in Fig.8. The NH_4-N production in the different media is shown in Fig. 9.

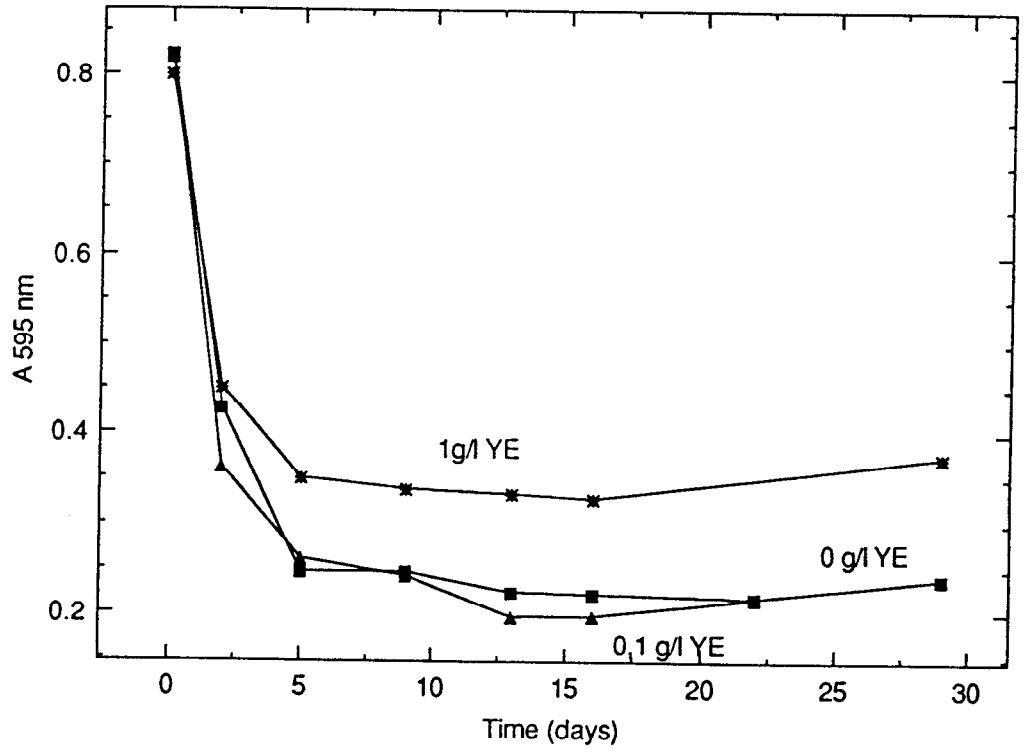

Figure 7. Gelatin degradation by *T. proteolyticus* in function of different YE concentrations.

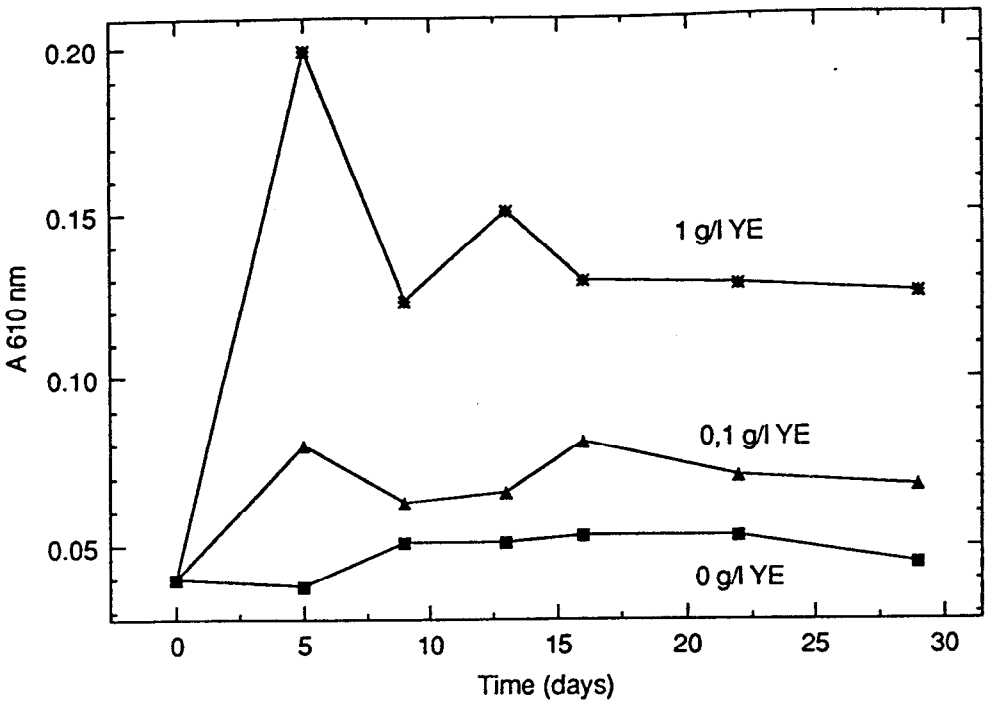

Figure 8. Growth of *T. proteolyticus* in media containing different concentrations of YE.

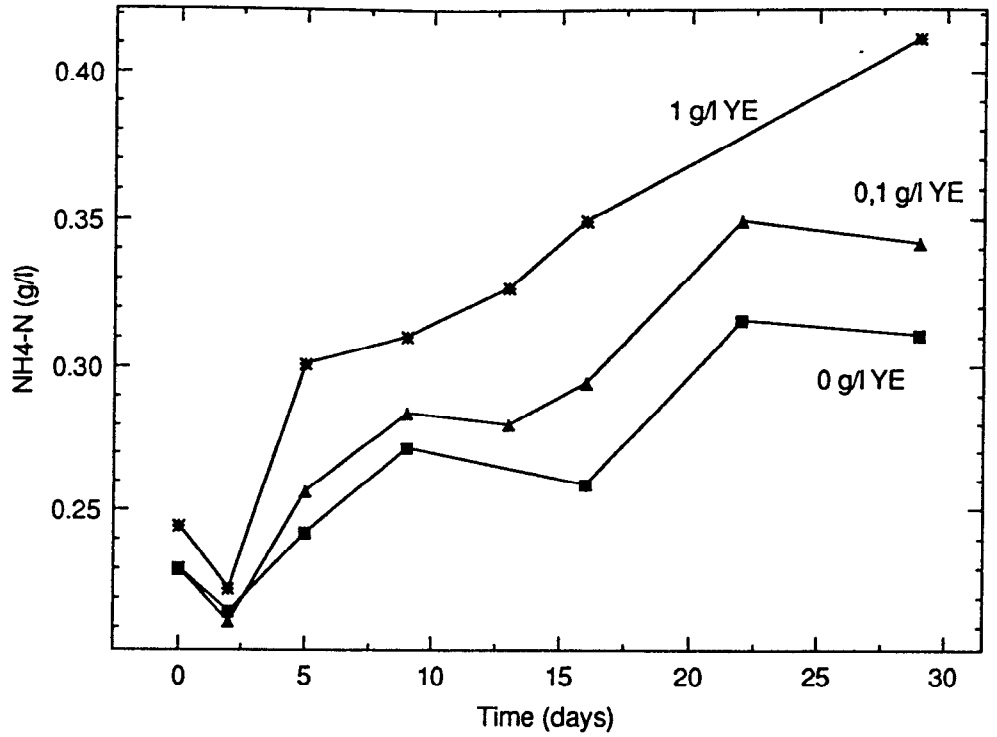

Figure 9. NH_4-N production by *T. proteolyticus* in media containing different concentrations of YE.

Table 5 compares growth of *T. proteolyticus* in function of different amounts of YE added to the medium. The N-balance before and after growth of *T. proteolyticus*is also represented in Table 5. The production of VFA in media with different NH_4Cl concentrations is shown in Fig. 10.

Table 5. Comparison of fermentation pattern and N-balance, before and after growth of *T. proteolyticus* in media containing different YE concentrations.

YE (g/l)	0	0,1	1
Growth (A 610 nm)	0,044	0,066	0,125
Kj-N (mg/l)	631,8	639,2	721,2
NH_4-N start (mg/l)	230	230	245
NH_4-N end (mg/l)	317	362	397
N_p (%) degraded	84	83	74
Org-N (%) hydrolyzed	22	32	32
ΔA 595 nm	0,140	0,155	0,296
Final pH	6,2	6,4	6,8
acetic a. (mg/l)	674	247	99
butyric a. (mg/l)	0	0	56
isobutyric a. (mg/l)	0	164	609
valeric a. (mg/l)	0	0	20
isovaleric a. (mg/l)	127	245	662
capronic a. (mg/l)	0	0	24
isocapronic a. (mg/l)	0	0	12
propionic a. (mg/l)	0	0	75

Experimental conditions: Products were measured after 36 days growth in rubber bung sealed flasks that contained 200 ml medium

with 0,3% substrate. All flasks were incubated at 60 °C under stationary conditions.

The organism grew poorly when no YE and 0,1 g/l YE was supplemented to the medium. Good growth of *T. proteolyticus* was observed when 1 g/l YE was added to the medium.
The degradation of gelatin ranged from 74% - 84%. The hydrolysis of gelatin diminished when 1 g/l YE was supplemented to the medium. When YE was added to the medium, almost 32% of the organic-N was completely mineralized towards NH_4-N.

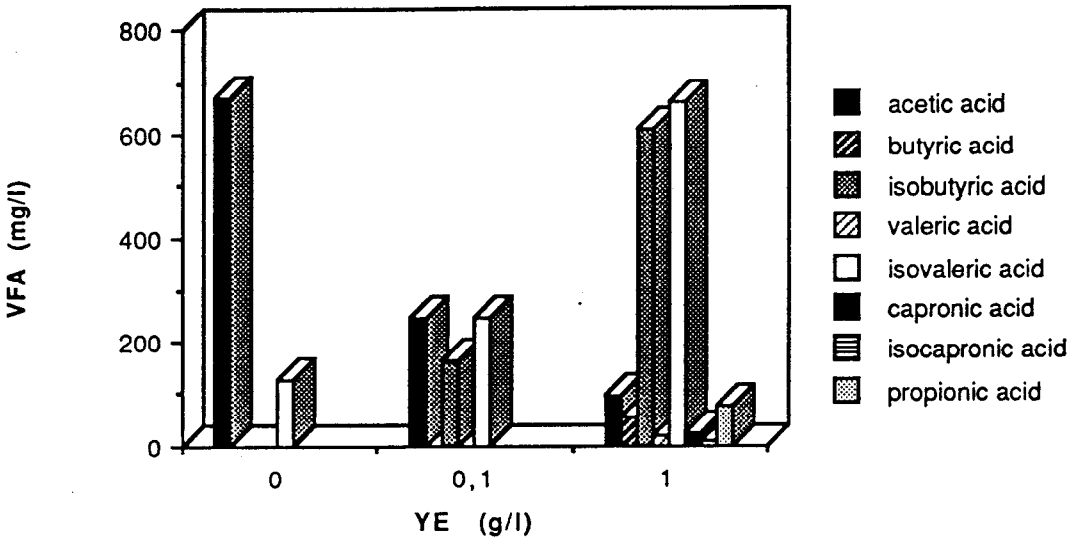

Figure 10. Production of VFA by *T. proteolyticus* in media with different YE concentrations.

The amount of isobutyric acid and isovaleric acid produced by *T. proteolyticus* increased when more yeast extract is supplemented to the medium. The production of acetic acid decreased when more yeast extract is added to the medium.

Conclusions

When different amounts of YE were supplemented to the medium, following observations could be made:

(1) *T. proteolyticus* grew poorly when no YE was added

(2) the same quantities of gelatin were degraded in a medium without YE and 0,1 g/l YE

As such, the fermentation of gelatin is optimal when 0,1 g/l YE is added to the medium.

4. Discussion

The study shows that *T. proteolyticus* can grow well on different protein sources. The best growth of the organism was obtained on the media containing gelatin and peptone as protein sources.

Gelatin is easy to handle and was therefore choosen as the protein substrate for studying more in detail the degradation of proteins by *T. proteolyticus*.

Gelatin was rapidly fermented by *T. proteolyticus*. The protein was not metabolised completely: 76% of the initial amount of gelatin was degraded, but only 26% of the organic-N was completely mineralized towards NH_4-N. Hence, a considerable part of the gelatin-N (ca. 70%) is converted to metabolite-N of unknown origin. The main products formed by *T. proteolyticus* were CO_2, H_2, acetic acid, ammonia and trace amounts of isobutyric acid and isovaleric acid. We shall try to regulate microbial metabolism to maximize the mineralization of the organic-N of unknown origin.

Ammonia inhibition is often considered as a cause of digestion failure. The effect of addition of different amounts of NH_4Cl (0 - 1 g/l) during anaerobic digestion of proteins was studied. No inhibition of gelatin degradation could be observed when NH_4Cl (0 - 1 g/l) was supplemented to the medium. There were more VFA produced when the amount of NH_4Cl supplemented to the medium increased. The growth of *T. proteolyticus* and the degradation of gelatin was not influenced by adding different amounts of NH_4Cl to the medium. As such, ammoniumchloride can be omitted.

Yeast extract was reported to be necessary for protein degradation (OLLIVIER *et al.*, 1985). Therefore, the influence of yeast extract on protein fermentation was examined. *T. proteolyticus* grew well when 1 g/l YE was supplemented to the medium, but growth was poorly in a medium without YE. Only 22% of the organic-N was mineralized in a medium without YE. When YE was added to the medium, almost 32% of the organic-N was completely mineralized towards NH_4-N. As such, an initial concentration of 0,1 g/l YE seems to be convenient in order to get the best mineralization of the protein.

The fermentation pattern of *T. proteolyticus*, in media containing different concentrations of gelatin will also be investigated.

We already obtained a few proteolytic, axenic strains on casein agar plates. However, when the strains were transferred to liquid medium, they did not grow. As such, the fermentation pattern of these strains could not yet been studied. The proteolysis by the different strains will be a further study objective. The degradative potential of *T. proteolyticus* and the isolated strains will be compared with each other in TN 15.3.

5. References

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