MELISSA

TN 15

TECHNICAL NOTES

TN 15. 1

MELISSA CCN 3

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TN 15.1

Isolation of proteolytic thermophilic bacteria

TN 15.1: ISOLATION OF PROTEOLYTIC THERMOPHILIC BACTERIA

1. Introduction

The input of biological polymers in the first compartment of the Melissa cycle is of critical importance; e. g. the crude protein concentration of faeces is estimated to be 20 -30 % of the total dry weight. In order to obtain an efficient cycling of N and S (and C) an extensive degradation of proteins is necessary. This degradation should not form dead end products nor metabolites toxic to the phototrophic bacteria of the second compartment. With respect to proteins, complete mineralisation of the amino acids with minimal build up of amines such as cadaverine and putrescine is of particular interest. To meet above mentioned demands, a detailed screening for proteolytic, thermophilic bacteria present in different anaerobic environments was set up.

In this study, we tried to isolate proteolytic, thermophilic bacteria from different anaerobic environments. Different isolation techniques were tested in order to obtain anaerobic, thermophilic, proteolytic bacteria.

2. Materials and methods

Inocula

Samples used for enrichment and isolation were obtained from:

(1) DRANCO (Dry Anaerobic Composting)
(2) compost
(3) hydrothermal springs of Kunashir Island (South Kurile Islands)
(4) intestinal tract of rats

Media

Media and culture methods used were appropriate for growth of stringent anaerobes. The composition of the different media, used for isolating proteolytic, thermophilic bacteria is shown in tables 1, 2, 3, 4, 5 and 6.

Table 1. Composition of Cooked Meat Medium (CMM)

CMM	100 g
gistextrakt	5 g
resazurine-oplossing (0,2 %)	0,5 ml
L-cysteïne.HCl (10 %)	5 ml
gedistilleerd water	1 l
eind-pH: 7,0	

Table 2. Composition of different media used for enrichment

	Soyton	Gelatine	Caseïne	Blanko
KH_2PO_4	1,5 g	1,5 g	1,5 g	1,5 g
K_2HPO_4	2,9 g	2,9 g	2,9 g	2, 9 g
$MgCl_2.6\ H_2O$	1,0 g	1,0 g	1,0 g	1,0 g
$CaCl_2.2\ H_2O$	0,15 g	0,15 g	0,15 g	0,15 g
$FeSO_4.6\ H_2O$	1,25 mg	1,25 mg	1,25 mg	1,25 mg
Bacto-Soyton	3 g			
Gelatine		3 g		
Caseïne			3 g	
Mineraal oplossing (PFENNIG & LIPPERT, 1966)	8 ml	8 ml	8 ml	8 ml
titriplex III 500 mg / l				
$FeSO_4.7\ H_2O$ 200 mg / l				
$ZnSO_4.7\ H_2O$ 10 mg / l				
$MnCl_2.4\ H_2O$ 3 mg / l				
H_3BO_3 30 mg / l				
$CoCl_2.6\ H_2O$ 20 mg / l				
$CuCl_2.2\ H_2O$ 1 mg / l				
$NiCl_2.6\ H_2O$ 2 mg / l				
$Na_2MoO_4.2\ H_2O$ 3 mg / l				
L-cysteïne.HCl (10 %)	5 ml	5 ml	5 ml	5 ml
resazurine-oplossing (0,2 %)	0,5 ml	0,5 ml	0,5 ml	0,5 ml
gedistilleerd water	1 l	1 l	1 l	1 l

Table 3. Composition of Thioglycollaat medium

pepton (gehydrolyseerde caseïne)	15 g
gistextrakt	5 g
L-cysteïne.HCl (10 %)	5 ml
NaCl	2,5 g
Na -thioglycollaat	0,5 g
resazurine-oplossing (0,2 %)	0,5 ml
gedistilleerd water	1 l
eind-pH: 7,1	

Table 4. Composition of Pepton-Yeast medium

proteose pepton		10 g
gistextrakt		10 g
zoutoplossing		40 ml
$CaCl_2$	0,2 g/l	
$MgSO_4$	0,2 g/l	
K_2HPO_4	1 g/l	
KH_2PO_4	1 g/l	
$NaHCO_3$	10 g/l	
NaCl	2 g/l	
resazurine-oplossing (0,2 %)		0,5 ml
L-cysteïne.HCl (10 %)		5 ml
gedistilleerd water		1 l
eind-pH: 7,2		

Table 5. Composition of gelatin medium

KH_2PO_4	1,5 g
K_2HPO_4	2,9 g
$MgCl_2.6\ H_2O$	1,0 g
$CaCl_2.2\ H_2O$	0,15 g
$FeSO_4.6\ H_2O$	1,25 mg
gelatine	3 g
mineraal oplossing (PFENNIG & LIPPERT, 1966)	8 ml
L-cysteïne.HCl (10 %)	5 ml
resazurine-oplossing (0,2 %)	0,5 ml

Table 6. Composition of bonch medium

NH_4Cl	0,33 g
KCl	0,33 g
$MgCl_2.2\ H_2O$	0,33 g
$CaCl_2.2\ H_2O$	0,33 g
KH_2PO_4	0,33
pepton	5 g
gistextrakt	0,1 g
mineraal oplossing (PFENNIG & LIPPERT, 1966)	1 ml
resazurine-oplossing (0,2 %)	0,5 ml
zwavel	10 g
gedistilleerd water	1 l

2.1. Enrichment of proteolytic, thermophilic bacteria from DRANCO

Two different enrichment procedures were used during the screening for proteolytic, thermophilic bacteria. These methods are shown in figures 1 and 2.

2.1.1. Enrichment method 1

One gram of DRANCO material was inoculated in 9 ml pepton-yeast medium (PY). The inoculum was incubated in a Bellco tube during 15 h at 60 °C. The experiment was continued as represented in fig. 1.

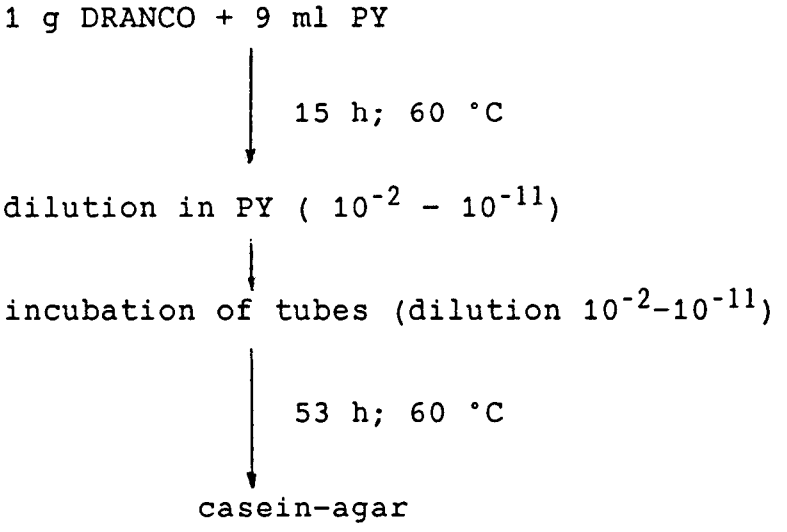

Figure 1. Scheme of the first enrichment method.

2.1.2. Enrichment method 2

Seven media, containing different protein sources were used fot the enrichment of proteolytic, thermophilic bacteria. The composition of the different media is shown in tables 1, 2, 3, 4, 5, and 6. DRANCO material (0,5 g) was inoculated in 200 ml of liquid medium. The flasks were incubated at 60 °C during different time intervals. The experiment was continued as represented in fig. 2.

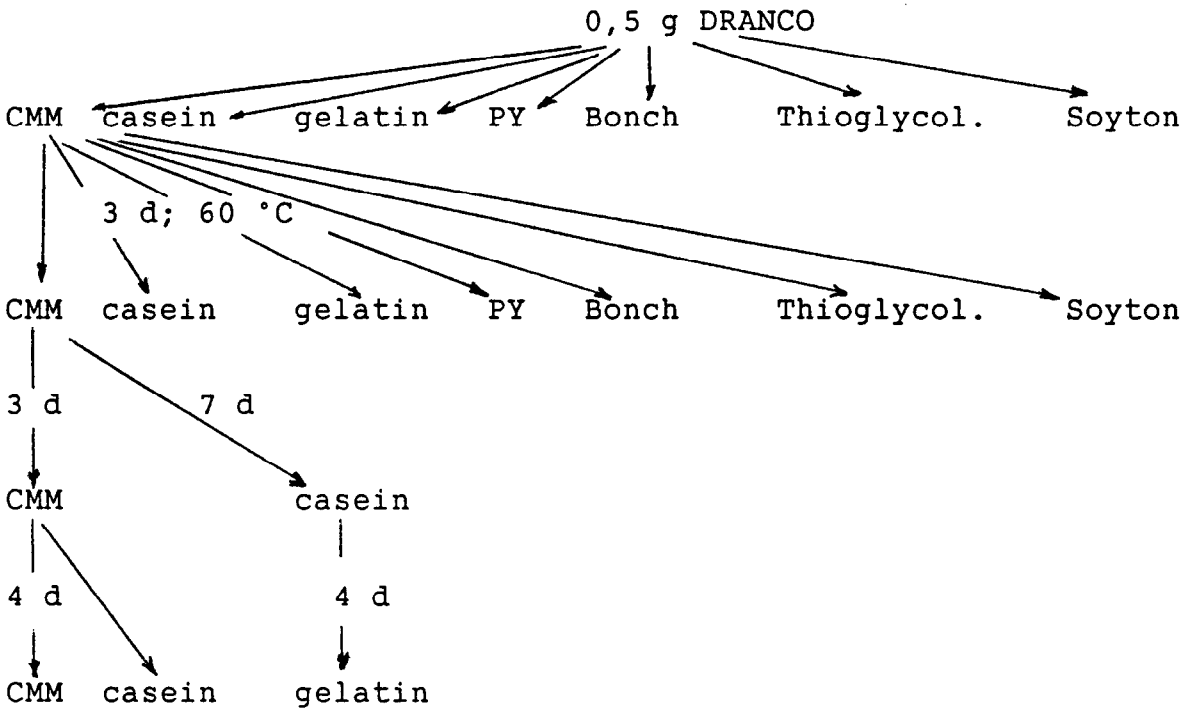

Figure 2. Scheme of the second enrichment method.

In fig. 2 the different transfers to other media are described, starting from CMM. The same procedure was followed for the samples inoculated in the other 6 media when growth occurred. There was growth when turbidity of the medium could be detected.

2.2. Isolation of proteolytic micro-organisms

2.2.1. Qualitatively assessment of proteolytic activity

Proteolytic activity of the cultures was screened **qualitatively** in four ways.

(1) casein was incorporated at 0,6 % (w/v) in agar plates which were then inoculated with the organism. Zones of clearing around the colonies after incubation were taken as evidence of proteolytic activity (fig 3.).

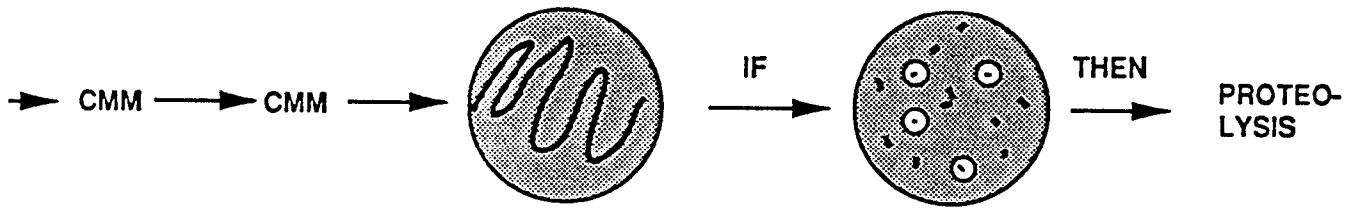

Figure 3. Clearing of casein-agar plates due to proteolytic activity.

(2) Cultures were grown in a medium comprising gelatin (12,5 % w/v) and liquefaction of the gelatin was monitored after several incubation periods. Since at the growth temperature (60 °C) used the medium was fluid, liquefaction was ascertained by testing for lack of solidification upon cooling to 5 °C during the growth period (fig. 4)

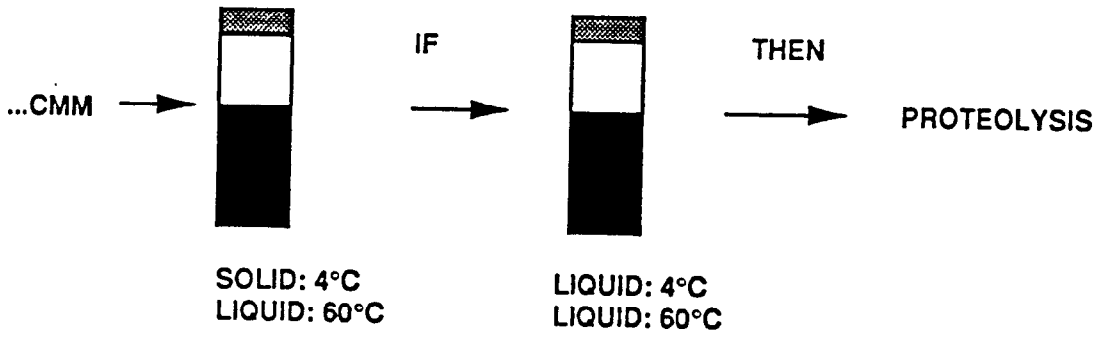

Figure 4. Liquefaction of gelatin at 5 °C.

(3) Liquid medium with casein (0,6 %) as sole carbon source

was inoculated with cultures and clearing was monitored for several days.

(4) The alkalinization of gelatin was determined in an anaerobic low-pepton basal medium (ALP). The ALP gelatin medium contained 0,002 % phenol red as indicator.

2.2.2. Semiquantitative assessment of proteolytic activity

Semiquantitative assessment of extracellular proteolytic activity was made with a modified casein agar plate method described by COWAR & DANIEL (1982). Samples from cultures were taken at different intervals during growth. The fluid was applied to wells cut into casein agar plates (0,6 % w/v casein). The plates were incubated at 60 °C for 1 week. Proteolytic activity was identified as circular zones of clearing around the cells (fig. 5). The diameter of the zones was proportional to enzyme activity. The casein agar plates were tested by using a known protease (Trypsine: 2 mg/ml) which was applied to the wells. Circular zones of clearing around the wells appeared after one day of incubation at 37 °C.

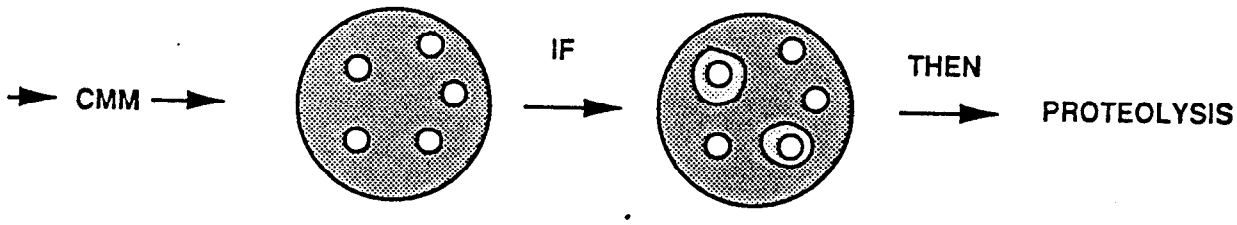

Figure 5. Semiquantitative assessment of proteolytic activity

3. Results

Proteolytic activity was detected by:

(1) blackening of Cooked Meat Medium

(2) lack of solidification at 5 °C in medium with gelatin

(3) clearing zones observed at casein agar plates

Blackening of CMM was observed after several transfers in following samples:

(a) CMM + 0,5 g DRANCO (24 d)

(b) CMM--->CMM--->CMM
3 d 4 d

(c) CMM---->CMM
20 d

(d) bonch---->CMM---->CMM----->CMM (4 d)
3 d 3 d 24 d

(e) casein---->CMM---->CMM---->CMM (18 d)
3 d 3 d 14 d

(f) soyton---->CMM---->CMM---->CMM (4 d)
3 d 3 d 14 d

Lack of solidification at 5 °C in medium with gelatin was observed in following samples (after several transfers and inoculating in Nutrient Gelatin):

(a) 1 g DR + 9 ml PY----->dilution 10^{-11}----->NG (21 d)
15 h 53 h

(b) 1 g DR + 9 ml PY----->dilution 10^{-2} in casein
15 h

----->NG (10 d)
6 d

(c) CMM----->CMM----->casein----->NG (26 d)
3 d 7 d 10 d

(d) CMM----->gelatin----->NG (19 d)
3 d 11 d

(e) bonch----->CMM----->NG (19 d)
3 d 11 d

(f) CMM----->NG (13 d)
20 d

(g) bonch----->CMM----->CMM----->CMM----->NG (22 d)
3 d 3 d 14 d 4 d

(h) CMM----->CMM----->NG (22d)
20 d 4 d

(i) casein----->CMM----->CMM----->CMM----->NG (22 d)
3 d 3 d 14 d 4 d

Eventually, clearing zones were observed on casein agar plates after several transfers to different media. The transfers needed for isolating proteolytic, anaerobic, thermophilic bacteria on casein agar plates are represented in next scheme.

(a) 1 g DR + 9 ml PY----->dilution 10^{-11}----->NG
15 h 53 h

----->cas-agar----->cas-agar----->cas-agar
50 d cl: 10 d cl: 7 d

gel-agar----->cas-agar (clearing: 7 d)
7 d

(b) 1 g DR +9 ml PY----->dilution 10^{-2} in casein
15 h

----->NG----->gel-agar----->cas-agar (cl: 7 d)
6 d 52 d 7 d

22 d

casein----->cas-agar----->cas-agar (cl: 7 d)
23 d 10 d

(c) CMM----->cas-agar----->cas-agar (cl: 7 d)
42 d 7 d

(d) bonch----->CMM----->CMM----CMM----->cas-agar
3 d 3 d 24 d 29 d

cl: 7 d

4 d

NG cas-agar

7 d

cas-agar cas-agar----->cas-agar (cl: 6 d)
cl: 7 d

11 d

cas-agar ALP-agar

10 d 14 d: red

cas-agar cas-agar (cl: 4 zones)

cl: 7 d

cas-agar (cl: 7 d)

4. Discussion

The study shows that there are anaerobic, proteolytic, thermophilic bacteria present in DRANCO. We were able to isolate some strains from DRANCO, but further purification on casein agar plates is still necessary.
Indications of proteolitic activity in enrichment samples from compost and hydrothermal springs of Kunashir Island were present. As such, further screening for proteolytic micro-organisms present in compost and hydrothermal springs would be interesting.

The degradative potential of the isolated strains will be investigated when axenic strains are obtained. The fermentation of proteins and subsequent metabolites formed by the isolated strains will be examined in order to get a better knowledge of the organic N-metabolism by anaerobic thermophilic micro-organisms. This knowledge will help us to use the metabolic potential of the isolated strains in the Melissa ecosystem. As such, a further examination of the fermentation pattern of proteins by proteolytic bacteria will be necessary.

5. References

COWAN, D. A. & DANIEL, R. M. (1982). Purification and some properties of an extracellular protease (caldolysin) from an extreme thermophile. Biochimica et Biophysica Acta, **704**, 293 - 305.