Growth and proteolytic activity of *Pseudomonas fluorescens* isolated from milk

Irena Rogelj

Original Scientific Paper - Izvorni znanstveni rad UDK: 579.23

Summary

The relation between growth kinetics of Pseudomonas fluorescens 33 and its proteolytic activity in raw and sterilized milk stored at 7° C was studied. Kinetics was determined by experimental growth curves. The proteolytic activity was measured by non-protein nitrogen (NPN), noncasein nitrogen (NCN) and tyrosine value (TV). The generation time of P. fluorescens 33 ranged from 7.9 hours to 11.1 hours and was shorter in sterilized milk. When population of P. fluorescens 33 reached stationary phase (18^8 CFU/ml) strong proteolytic activity was observed. During six days of storage the amount of NCN increased from 120 mg/100 g to 280 mg/100g.

Additional index words: Pseudomonas fluorescens activity, raw milk, sterilized milk, proteolytic activity, milk storage (7° C).

Introduction

Psychrotrophic bacteria, particularly *Pseudomonas spp.* have been implicated in the spoilage of milk and dairy products because of their ability to produce extracellular proteases and lipases. These enzymes are sufficiently heat stable to remain active after pasteurization even though the organisms themselves are killed. Relatively small amounts of enzyme present in the raw milk may result in the appearance of proteolysis and lipolysis in the final product. Some of these proteases can increase both noncasein and nonprotein nitrogen fractions in milk (Bishop and White, 1986; Ellis and Marth, 1984; Fairbairn and Law, 1986; Fernandez et al., 1992; Miranda and Gripon, 1986).

Since the activity of psychrotrophic microorganisms causes uncontrolled protein degradation, the enzymatic activity of psychrotrophs is of great interest.

The aim of our research was, therefore, to study the growth kinetics of proteolytic isolate in raw and sterilized milk as well as to find out at what size and stage of growth the proteolytic activity begins.

Materials and methods

The milk used in study was collected by machine milking of a single cow in the third lactation. The representative amount from the total milk was divided to samples which were named according to later treatment as:

M	sample of raw milk,
SM	sample of sterilized milk,

$M+33_a$, $M+33_b$	samples of raw milk inoculated with isolate at two population size,
$SM+33_a$, $SM+33_b$	samples of sterilized milk inoculated with isolate at two population size,
M+T	sample of raw milk to which bacteriostatic agent Thimerosal (Sigma Chemical Co.) was added in concentration of 0.01 w/v.

The bacteria used to inoculate samples of milk was one of isolates isolated from raw and pasteurized milk. From 51 proteolytic bacteria which were isolated, the isolate 33 was selected for inoculation because of its intensive proteolytic activity at 7 °C. On the basis of identification tests with API 20 NE system, the isolate was identified as *Pseudomonas fluorescens* strain and we named it as *P. fluorescens 33*. Before inoculation the isolate was grown on nutrient broth (Difco) at 31 °C for 18 hours. The level of inoculant used was such that samples of raw and sterilized milk contained 3×10^4 CFU/ml ($M+33_a$ and $SM+33_a$) or 3×10^5 CFU/ml ($M+33_b$ and $SM+33_b$). The milk samples were incubated at 7 °C.

In order to follow the bacterial growth and proteolysis, all milk subsamples were analyzed on the day of incubation and at 2-day intervals thereafter throughout the 10-day storage period. Bacterial count (CFU/ml) was made on milk agar according to the procedures in Standard Methods for the Examination of Dairy Products (1978). Plates were incubated at 31 °C for 48 hours. The degree of proteolysis was estimated by non-protein nitrogen (NPN), non-casein nitrogen (NCN) (Methodenbuch Band VI, 1985) and tyrosine value (TV) (Juffs, 1973).

To determine the growth kinetics of microbial population, the generation times calculated from experimental growth curves were used.

Results and discussion

Growth of P. fluorescens 33

The native microbial population of raw milk (6×10^3 CFU/ml), in which *P. fluorescens 33* was inoculated in two different concentrations (3×10^5 CFU/ml and 3×10^4 CFU/ml), was 50-fold and 5-fold smaller than inoculated *P. fluorescens 33* population. Therefore in inoculated raw milk *P. fluorescens 33* was the dominating microbial population.

An exponential growth of *P. fluorescens 33* immediately after inoculation in raw and sterilized milk was observed (fig. 1).

The shorter generation time was found in sterilized milk in both initial size of population. The generation time was shorter in samples inoculated with 3×10^4 CFU/ml than in samples inoculated with 3×10^5 CFU/ml (Tab. 1). The generation time around 8 hours for the 7 psychrotrophic *Pseudomonas strains* were found also by Patel et al. (1983) and for *P. fluorescens* by Fernandez et al. (1992). Shorter generation time (4 hours) was found by Sanjose et al. (1987) for the *P. fluorescens NCDO 2085*.

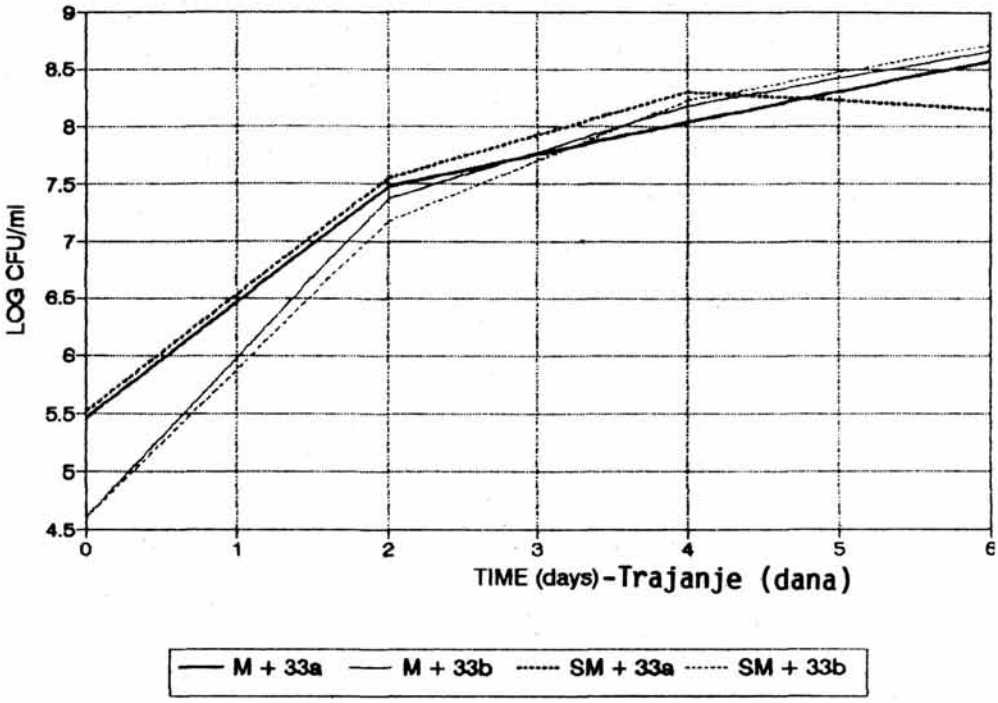

Fig. 1: Growth of *P. fluorescens 33* in raw and sterilized milk at 7° C

Sl. 1: Rast *P. fluorescens 33* u sirovom i steriliziranom mlijeku pri 7° C

Table 1. The population kinetic parameters of *P. fluorescens 33* in raw and sterilized milk

Tablela 1. Parametri kretanja populacije *P. fluorescens* u sirovom i steriliziranom mlijeku

uzorak sample	k	G*
$M+33_a$	0.089	11.1
$M+33_b$	0.124	8.1
$SM+33_a$	0.096	10.4
$SM+33_b$	0.126	7.9

* generation time — generacijsko vrijeme

Proteolytic activity

The lowest proteolytic activity was observed when bacteriostatic agent Thimerosal was added to raw milk immediately after milking (Tab. 2, 3 and 4).

Therefore, it can be concluded that native proteolytic activity was law.

Table 2. NPN content (in mg/100 g) in different samples of raw and pasteurized milk stored at 7 °C
Tabela 2. Količina NPN (mg/100 g) u različitim uzorcima sirovog i pasteriziranog mlijeka skladištenog pri 7 °C

sample uzorak	time of storage (days) — skladištenje (dana)					
	0	2	4	6	8	10
M	24	21	26	24	26	28
M+T	24	24	31	25	28	28
$M+33_a$	24	21	35	89	C*	
$M+33_b$	24	25	32	69	C*	
$SM+33_a$	28	25	34	79	100	125
$SM+33_b$	28	26	32	55	89	101

* coagulation — koagulacija

Table 3. NCN content (in mg/100g) in different samples of raw and pasteurized milk stored at 7 °C
Tabela 3. Količina NCN (mg/100g) u različitim uzorcima sirovog i pasteriziranog mlijeka skladištenog pri 7 °C

sample uzorak	time of storage (days) — skladištenje (dana)					
	0	2	4	6	8	10
M	120	126	129	136	140	151
M+T	120	120	120	124	127	127
$M+33_a$	120	124	134	285	C	
$M+33_b$	120	124	116	267	C	
$SM+33_a$	42	43	105	202	220	241
$SM+33_b$	42	43	75	144	189	220

Table 4. TV (in mg of tyrosine/ml) in different samples of raw and pasteurized milk stored at 7 °C
Tabela 4. TV (mg tirozina/ml) u različitim uzorcima sirovog i pasteriziranog mlijeka ohlađenog pri 7 °C

sample uzorak	time of storage (days) — skladištenje (dana)					
	0	2	4	6	8	10
M	0.63	0.52	0.66	0.69	0.67	0.83
M+T	0.63	0.57	0.59	0.56	0.64	0.64
$M+33_a$	0.63	0.53	0.70	0.88	C	
$M+33_b$	0.63	0.68	0.74	0.84	C	
$SM+33_a$	0.33	0.27	0.51	0.71	0.83	0.90
$SM+33_b$	0.33	0.28	0.45	0.63	0.70	0.81

The steady but relatively law degree of milk proteolysis, caused by native microbial population, was noticed from the start to the eighth day of the storage. The amount of CN during this period showed a constant decline (0.8%/2 days). From the eight to tenth day of the storage the CN dropped by 1.8%.

Table 5. Casein-nitrogen content (in % of total nitrogen) in different samples of raw and pasteurized milk stored at 7 °C

Tabela 5. Količina kazeinskog dušika (u % ukupnog dušika) u različitim uzorcima sirovog i pasteriziranog mlijeka skladištenog pri 7 °C

sample uzorak	time of storage (days) — skladištenje (dana)					
	0	2	4	6	8	10
M	80.2	79.2	78.7	77.6	76.9	75.1
M+T	80.2	80.2	80.2	79.5	79.0	79.0
$M+33_a$	80.2	79.5	77.9	53.0	C	
$M+33_b$	80.2	79.5	80.9	55.9	C	
$SM+33_a$	93.2	93.0	83.0	67.3	64.4	61.0
$SM+33_b$	93.2	93.0	87.9	76.7	69.4	64.4

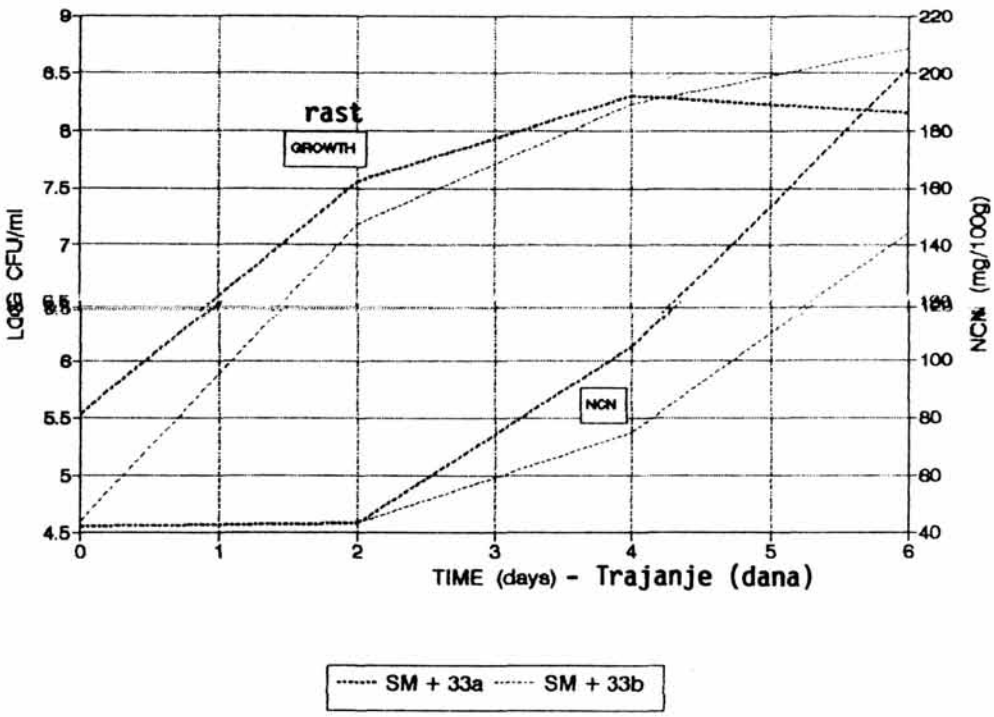

Fig. 2. Growth and proteolytic activity of *P. fluorescens 33* in raw milk at 7 °C

Sl. 2. Rast i proteolitička aktivnost *P. fluorescens 33* u sirovom mlijeku pri 7 °C

The largest degree of proteolysis was found in samples inoculated with *P. fluorescens 33*. Intensive synthesis of proteinases started after the inflection point of exponential growth. At this stage the population size od *P. fluorescens 33* ranged from 1.5×10^7 to 3.2×10^7 CFU/ml. Fast protein degradation which followed intensive synthesis of proteinases reached maximum between forth and sixth day of the storage. At this time population size of isolate was approx. 10^8 CFU/ml (Fig. 2 and 3). The results are in accordance with results obtained by Rowe and Gilmour (1982), McKellar and Cholette (1984) and Griffiths (1989).

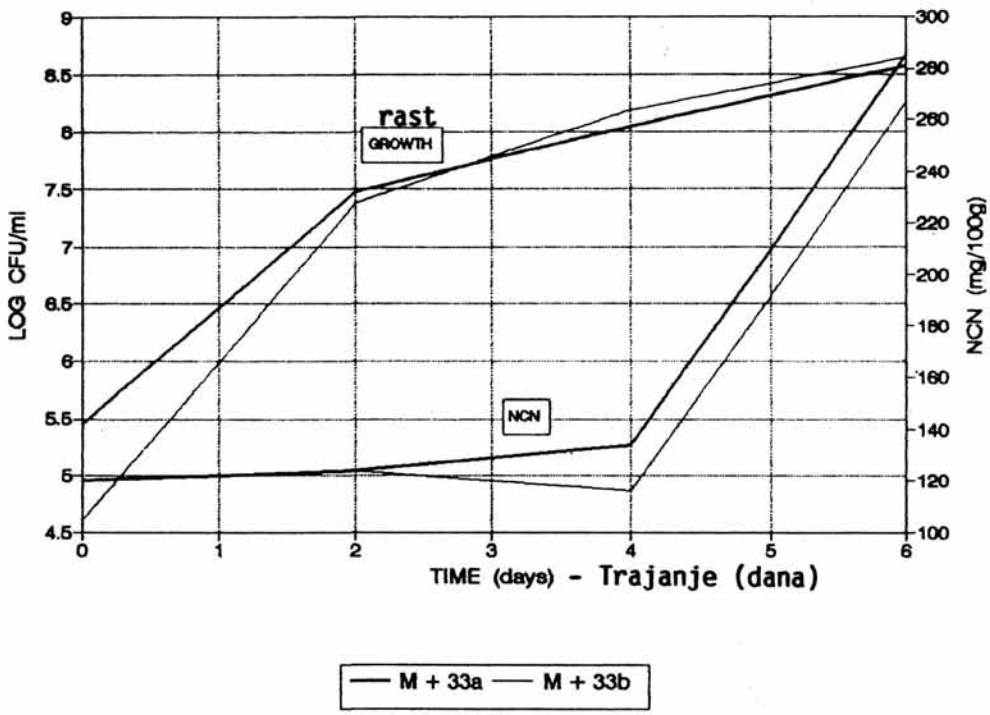

Fig. 3. Growth and proteolytic activity of *P. fluorescens 33* in sterilized milk at 7 °C
Sl. 3. Rast i proteolitička aktivnost *P. fluorescens 33* u steriliziranom mlijeku pri 7 °C

Conclusion

Fast protein degradation was observed when the stationary phase of *P. fluorescens 33* growth hat been reached. At this time, population size of isolate was approx. 10^8 CFU/ml. Three differences were found when comparing the activity of *P. fluorescens 33* in the sterile and raw milk. The generation time was shorter in sterilized milk. The fast inorease of NCN and TV was observed in raw milk between forth and sixth day of the storage, but in sterile

milk already after two days of the storage, probably due to the reversibility of casein-whey protein complex. At the same degree of proteolysis the coagulation appeared only in raw milk.

RAST I PROTEOLITIČKA AKTIVNOST PSEUDOMONAS FLUORESCENS IZOLIRANIH IZ MLIJEKA

Sažetak

Istraživan je odnos između brzine rasta i proteolitičke aktivnosti Pseudomonas fluorescens 33 u sirovim i steriliziranom uskladištenom mlijeku (7°C). Brzina rasta određena je eksperimentalnim krivuljama rasta. Proteolitička aktivnost mjerena je određivanjem količina neproteinskog dušika, nekazeinskog dušika i vrijednosti tirozina. Generacijsko vrijeme Pseudomonas fluorescens 33 kretao se od 7,9 sati do 11,1 sat i bilo je kraće u steriliziranom mlijeku. Kada je populacija Pseudomonas fluorescens 33 dostigla fazu mirovanja (10^8 jedinica koje formiraju kolonije/ml) uočena je intenzivna proteolitička aktivnost. Tijekom 6 dana skladištenja koliličina nekazeinskog dušika povećala se od 120 mg/100 g do 280 mg/100 g.

Riječi natuknice: Aktivnost Pseudomonas fluorescens, sirovo mlijeko, sterilizirano mlijeko, proteolitička aktivnost, skladištenje mlijeka (7°C).

References

BISHOP, J. R., C. WHITE (1986): Assessment of Dairy Product Quality and Protential Shelf-Life — A Review. **Journal of Food Protection, 49** (9) p. 739—753.

ELLIS, B. R., E. H. MARTH (1984): Growth of *Pseudomonas* or *Flavobacterium* in Milk Reduce Yield of Cheddar Cheese. **Journal of Food Protection, 47** (9) p. 713—716.

FAIRBAIRN, D. J., B. A. LAW (1986): Proteinases of psychrotrophic bacteria: their production, properties, effects and control. **Journal of Dairy Research, 53** (1) p. 139—177.

FERNANDEZ, L., A. JASPE, A. ALVAREZ, P. PALACIOS, C. SANJOSE (1992): Proteolytic and lipolytic activities of *Pseudomonas fluorescens* grown in raw milk with variable iron content. **Milchwissenschaft, 47** (3) p. 160—163.

GRIFFITHS, M. W. (1989): Effect of temperature and milk fat on extracellular enzyme synthesis by psychrotrophic bacteria during growth in milk. **Milchwissenschaft, 44** (9) p. 539—543.

JUFFS, H. S. (1973): Proteolysis detection in milk. Interpretation of tyrosine value data for raw milk supplies in relation to natural variation, bacterial counts and other farctors. **Journal of Dairy Research, 40** p. 371—381.

McKELLAR, R. C., H. CHOLETTE (1984): Synthesis of Extracellular Proteinase by *Pseudomonas fluorescens* Under Conditions of Limiting Carbon, Nitrogen, and Phosphate. **Applied and Environmental Microbiology, 47** (6) p. 1224—1227,

METHODENBUCH BAND VI (1985): Chemische, physikalische und mikrobiologische Untersuchungs-verfahren für Milch, Milchprodukte und Mölkereihilfsstoffe. Darmstadt, VDLUFA — Verlag, C 30.2, C 30.3, C 30.4.

MIRANDA, G., J. C. GRIPON (1986): Origine, nature et incidences technologiques de la proteolyse dans le lait. **Le Lait, 66** (1) p. 1—18.

PATEL, T. R., F. M. BARLETT, J. HAMID (1985): Extracellular Heat-Resistant Proteases of *Psychrotrophic Pseudomonas*. **Journal of Food Protection, 46** (2) p. 90—94.

ROWE, M. T., A. GILMOUR (1982): Growth, enzyme production and changes in oxygen tension occurring during batch cultivation of psychrotrophic *Pseudomonas fluorescens* strains. **Milchwissenschaft, 37** (10) p. 597—600.

SANJOSE, C., L. FERNANDEZ, P. PALACIOS (1987): Compositional Changes in Cold Raw Milk Supporting Growth of *Pseudomonas fluorescens NCDO 2085* before Production of Extracellular Proteinase. **Journal of Food Protection, 50** (12) p. 1004—1008.

Standard Methods for the Examination of Dairy Products (1978): Washington, American Public Health Association, p. 77—95, p. 107—115.

Adresa autora — Author's address:
Assist. prof. dr. Irena Rogelj
Biotehniška fakulteta, Institut za mlekarstvo
Domžale, SLO

Primljeno — Received
20. 11. 1992