Protease and Lipase production of psychrotrophic bacteria of dairy origin

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SUMMARY

Psychrotrophic bacteria were isolated from raw milk samples by incubation at 5°C — 7°C. Viable counts of these bacteria were carried out and visible colony forming units were found after 7days and 10 days at 5°C — 7°C. Isolates were tested for production of the extra-cellular enzyme lipase and protease. In each case enzyme positive cultures were purified and tested to identify to a generic level. The optimum temperature for enzyme production was tested for and was generally 30°C. The temperature at which the organisms were grown had effect on enzyme production.

INTRODUCTION

A trend was observed in storage of raw milk at refrigeration temperature for two days or longer on the farm and at the processing plants prior to heat treatment. Psychrotrophs become the predominant micro flora of the raw milk. Psychrotrophs can be defined as the microorganism having the ability to grow rapidly at refrigeration temperature (3°C-7°C) irrespective of the known optimum temperature ranging between 20°C — 30°C (Marshall, 1979). Most of psychrotrophs bacteria are gram negative rods, non-spore formers of the genera, *Pseudomonas, Flavobacterium, Alcaligenes, Entrobacteriaceae* and *Chromo-bacterium* spp (Murray and Steward, 1978; Muir *et al.*, 1979). Sporing and non-sporing gram-positive organisms both have also been isolated and these were *Bacillus, clostridium, Micrococcus, Corynebacterium, Streptococcus* and *Arthrobacter* spp (Collins, 1981; Griffiths *et al.*, 1981; Johnson and Bruce, 1982).

Most of psychrotrophs bacteria are destroyed by pasteurization (except spore formers), never-the-less they produce extra cellular enzymes, which are extremely, heat stable (Sorhaug and Stepaniak, 1991, Champagne *et al.*,

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1994; Zahran and Ward, 2001). Thus, the microbial enzymes, which survive the heat treatment, can affect the quality of many heat-treated dairy products by causing deterioration of flavor during the storage of the products (Fitzratrick, 2001).

The objectives of this study were to isolate proteolytic and lipolytic bacteria of dairy origin and to study their enzymes activity.

MATERIALS AND METHODS

Incubation of the milk sample:

A raw milk sample was incubated in a cold room with temperature ranging from 5°C to 7°C for seven days then the following tests were carried out.

Estimation of viable count: proteolytic and Lipolytic counts using the surface plate method as reported by Luck (1972). A total viable count for all colonies and counts of proteolytic or lipolytic colonies were carried out on skimmed milk agar and Victoria blue margarine agar respectively.

Identification of the isolates: proteolytic and lipolytic isolates were classified according to Bergey's manual of determinative bacteriology (Buchman and Gibbson, 1974) and the Gram negative rod-shaped bacteria were identified using the first stage diagnostic procedure of Cowan and Steel (1965).

Enzyme production:

The method described by Griffiths *et al.,* (1981) was used for production of protease and lipase enzymes. Organisms were grown in Skerman's mineral salts medium at 30°C, 25°C, and 4°C for fourteen days. The supernatants from these cultures were used as a source of Protease and Lipase enzymes.

Enzyme Assay:

Lawrence *et al.,* (1967) agar diffusion method was adopted with slight modifications. The activity of the enzymes was tested in 30% skim milk agar as a substrate for protease and tributyrin agar for Lipase. The substrate medium was poured as a thin layer for protease and over nutrient agar for lipase. Holes 4mm in diameter were cut in the agar with a sterile cork borer. 20111 of enzyme sample were placed in the holes using a micro syringe. Five holes were used per isolate / temperature (the diameter of the zone is an average of five holes). The plates were covered and incubated at 30°C for 7 days. Enzyme activity appeared as a clear zone or a precipitation around the holes. The diameters of the zones were measured.

RESULTS AND DISCUSSION

Total viable counts and proteolytic and Lipolytic counts were recorded after ten days incubation at 5°C — 7°C. (Table1). Percentage of the total viable count to proteolytic count, lie between 14% — 15% and total viable count to lipolytic count between 10% — 11% almost similar. However, it appears from the result obtained, psychrotrophic counts of more than 106 cfu /ml in raw milk can result in production of these enzymes. Matselies and Rouissis (1998) observed maximum protease and lipase activities when bacterial count were 108 — 109 and 107 — 108 cfu /ml respectively. Psychrotrophic microorganisms isolated from raw milk were Alcaligenes, pseudomonas, Bacillus spp. and Entrobacteriaceae. Pseudomonas spp. was found to be the predominant species synthesizing these enzymes. From the four organisms isolated Bacillus spp. showed the highest protease activity of all four organisms. The highest activity was observed when the organism was grown at 30°C and enzyme tested at the same degree of temperature. When the organism was grown at 30°C and the enzyme was tested at 25°C, the drop in activity of protease was only 0.6%. A high drop in activity (42.7%) was observed when the organism was grown at 30°C and enzyme tested at 4°C. so the temperature at which the enzyme was tested appeared to have a bearing on the enzyme activity. Adams et al., (1975) observed that optimum temperature for protease activity of different psychrotrophic bacteria lay between (40°-45°C). This explains why protease of four isolates tested showed a high activity at 30°C. From these results (Table 2 and 3) it can be assumed that optimum enzyme activity is related to optimum growth temperature. It can also be assumed that the *Bacillus* spp. is a high mesophilic psychrotrophs, although growing and producing protease at 4°C would appear to grow better and produce more protease at 25°C with the best at 30°C. From this assumption the Entrobacteriaceae could be termed a mid, mesophilic psychrotrophs, because its best activity was at the 25°C level. Similarly the *Pseudomonas* spp. (organism 2) could be considered a psychrophilic psychrotrophs since its best activity was observed at 4°C.

Table 1. Proteolytic, lipolytic and total viable count (in 30% skim milk and Victoria blue Margarine agar) of psychrotrophic bacteria incubated at 5°C-7°C for? and 10 days .

Test	Incubation time (days)	
	7	10
	logiocfu/ml	logiocfu/m1
Total viable count/m1 (30% skim milk)	7.150	7.184
Proteolytic count/m1	NC	6.223
Total viable count/ml (Victoria blue margarine agar)	NC	7.167
Lipolytic count/nil	NC	6.173

NC: Not counted (>300)

Table 2. Protease activity of different psychrotrophic bacteria .

Temperature at which organisms incubated and lipase produced (˚C)	Temperature at which lipase was tested (°C)	Enzyme activity (diameter of zone in mm) for organisms			
		Pseudomonas sPP (*)	*Pseudomonas* sPP (*)	*Bacillus* spp	Entrobacteriaceae
30	30	31.25	12.25	39.25	26.60
	25	30.33	10.50	39.00	21.00
	4	7.95	5.50	22.50	9.60
25	30	17.25	8.30	36.00	31.30
	25	19.00	15.50	34.00	22.30
	4	15.00	5.50	24.00	24.50
4	30	29.30	15.50	9.60	9.60
	25	23.00	15.50	10.00	9.50

* *Number shown is an average of 10 readings .*

For lipase activity *Pseudomonas* spp (organism 3) would appear to be the least active lipase producer of the four isolates but in terms of activity the results showed little variation over the three temperatures at which the isolates were grown (Table 3). Using the same assumption of the connection between enzyme and optimum growth temperature the *Pseudomonas* spp. (organism 4) would appear to be a mesophilic psychrotrophs, as like the *Alcaligenes* spp. The Entrobacteriaceae (organism 2) tends to be a psychrophilic Psychrotroph, while the other *Pseudomonas* spp (organism 3) did not fit into this pattern. From Table (3) lipase activity was better at 25°C and 30°C than at 4°C. Those findings were opposite to the observation of Griffiths (1989) who reported that a maximum lipase activity at 5°C was similar to that at 25°C.

As conclusions, the effective control of Psychrotrophs must begin on the farm and be followed through all the way till the retail stores. Clean equipment and packages, limited time of storage and low holding temperature for raw milk will lower the growth of Psychrotrophs.

Table 3. Lipase activity of different psychrotrophic bacteria.

Temperature at which organisms incubated and lipase produced (°C)	Temperature at which lipase was tested (°C)	Enzyme activity (diameter of zone in mm) for organisms			
		Alcaligenes spp	*Pseudomonas* spp (3)	Enterobacteriaceae (2)	*Pseudomonas* spp (4)
30	30	11.00	8.00	9.00	15.25
	25	15.50	8.75	7.75	14.25
	4	13.60	5.00	8.50	9.25
25	30	12.25	7.75	20.60	8.00
	25	12.00	5.30	16.00	13.00
	4	7.50	5.00	9.50	8.25
4	30	11.00	8.00	17.30	7.00
	25	6.60	7.00	19.00	6.30
	4	7.00	4.30	16.00	6.00

Number shown is an average of 10 readings

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REFERENCES

Adams, D.M., Barach, J.T. and Speck, M.L. (1975). Heat resistant protease produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Science, 58, 828-834.

Buchanan, R.E.; and Gibbsons, N.E. (1974). Bergey's Manual of Determination bacteriology, 8th ed. Baltimore. The Williams and Wilkins.

Champagne, C.P; Lating, R.R.; Roy, D.; Mafu, A.A. and Griffiths, M.W. (1994). Psychrotrophs in dairy products: Their effect and their control. Critical review of food Science and Nutrition 34: 1-30.

Collins, E.B. (1981). Heat resistant psychrotrophic microorganism. J. Dairy Sci., 64: 157-196.

Cowan, S.T. and Steel, K. J. (1966). Manual for the Identification of Medical Bacteria. Cambridge, Cambridge University Press.

Fitzpatrick, K. (2001). The effect of raw milk handling on lipolysis in milk and whole milk powder and implication for product quality. M. Sc. thesis, University of Melbourne, Australia.

Griffiths, M.W.; Phillips, J.D. and Muir, D.D. (1981). Thermo stability of proteases and Lipases from a number of species of psychrotrophic bacteria of dairy origin, J. Applied Bacteriology. 52: 333-337.

Griffiths, M.W. (1989). Effect of temperature and milk fat on extra cellular enzyme synthesis by psychrotrophic bacteria during growth in milk. Milckwisscensschaft, 44,539-543.

Johnson, D.W. and Bruce, J. (1982). Incidence of thermo uric psychrotrophs in milk produced in the west of Scotland, J. Applied Bact. 52: 333-337.

Lawrence, R.C.; Fryer, T.F. and Reiter, B. (1967). Rapid method for the quantitative estimation of microbial lipases. Nature, London, 213, 1264-1265.

Luck, H. (1972). Bacteriological quality test for bulk cooled milk. Dairy Science Abstract, 34: 101-122.

Marshal, R.T. (1979). Psychrotrophic bacteria their relationship to raw milk, quality and keeping quality of cottage cheese. In Marshal Italian and specially cheese seminars, Italy.

Matselis, E. and Roussis, G.L. (1998). Proteinase and lipase production by *Pseudomonas fluorescents,* proteolysis and lipolysis in the rmized ewe's milk. Food Control 9(5), 215-259.

Muir, D.D.; Phillips, J.D. and Dalgleish, D.G. (1979). The Lipolytic and Proteolytic activity of bacteria isolated from blended raw milk. J. of the Society of Dairy Techno. 32: 19-23.

Murray, J.G. and Stewart, D.B. (1978). Advances in the microbiology of milk and dairy products. J. of Society of Dairy Techno. 31: 28-35.

Sorhaug, T. and Stepaniak, L. (1991). Microbial enzymes in the spoilage of milk and dairy products .In: Food Enzymology, vol.4, P.E. Fox (Ed) pp 169-218. Elsevier Applied Science. U.S.A.

Zahran and ward (2001). Synthesis of heat stable extra cellular proteinase by *Pseudomonas fluorescens* R8 isolated from raw milk. http:// ift. confex. Com/ift/2001/tech program /paper 6507. htm.

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