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Influence of culture conditions on esterase activity of five psychrotrophic Gram negative strains selected from raw Tunisian milk

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Abstract - The contamination of milk by spoilage bacteria is undesirable, particularly when Gram negative bacteria which produce thermo-resistant protease and lipase can grow. In this work, spoilage bacteria in refrigerated raw milk were identified, using API 20NE System. Five dominant species were found: *Pseudomonas fluorescens* (20%), *Aeromonas hydrophila* (16%), *Pseudomonas cepacia* (13%), *Pseudomonas putida* (6%) and *Chryseomonas luteola* (5%). On the basis of agar diffusion assays, five strains harbouring the strongest lipases activities were selected. It has been found that esterase activities are higher for each one. Effects of main environmental and nutritional factors on the esterase activity of those psychrotrophic strains were investigated. Biomass level, pH, lactose concentration and permanent agitation affected positively esterase activity of each strain. However, the addition of Tween 20 influenced it negatively. Finally, and in order to extract information from the data sets, principal components analysis was applied to the data sets. The first two principal components showed a clear discrimination between *Pseudomonas fluorescens* and *Pseudomonas cepacia*.

Key words: raw milk, Gram negative bacteria, esterasic activity, optimisation.

INTRODUCTION

The quality of raw milk and dairy products has been considerably improved by refrigeration on farms and in processing plants. Unfortunately, the current practices for the collection and storage of raw milk favoured the growth of psychrotrophic bacteria, able to grow at a temperature below 7 °C. *Pseudomonads* are the most common group in raw milk at the time of spoilage. Significant contaminations by these microorganisms occur due to inadequately sanitised surfaces of milking, storage and transportation equipment (Dousset *et al*., 1986; Aaku *et al*., 2004). Besides their rapid growth ability in refrigerated milk, psychrotrophs produce heat-stable extra-cellular proteases, lipases and phospholipases: some enzymes can survive pasteurisation and even UHT heat treatments (Dousset *et al*., 1988, Braun *et al*., 1999). *Pseudomonas* spp. is the primary concern with regard to lipolytic degradation of milk (Shah, 1994; Rahman *et al*., 2005). Ester-hydrolases, including esterase and lipase, by hydrolysing triglycerides and esterification of certain low molecular weight fatty acids with ethanol, produce flavour defects associated with fat breakdown in cream, butter, cheese, and UHT products. In fact, most of the lipases retain some of their activities after pasteurisation and even after UHT treatment (Deeth, 2002). Proteases are associated with bitterness in milk, jellification of UHT sterilised milk, and reduced yields of soft cheese. Most of the proteases are able to degrade κ^-, α_{s1} and β-caseins; they are remarkably heat stable (Miranda and Gripon, 1986; Mc Phee and Griffiths, 2002).

The objective of the present study is to characterise some of the spoilage Gram negative psychrotrophs present in raw milk on the lipolytic activities. Another goal is to determine the effect of medium composition (concentration of Tween 20, concentration of lactose, bacterial biomass, concentration of potassium phosphate) and environmental characteristics (pH, temperature of incubation, agitation) of the most active psychrotrophic bacteria on esterase activity, by using experimental design (matrix with seven factors) and Principal Components Analysis (PCA).

MATERIALS AND METHODS

Sampling. Forty samples of refrigerated milk were obtained from ten collection centres situated in northern and central Tunisia during the spring period when the lactation curve was at its maximum. All collection centres have refrigerated vans between 4 and 6 ºC.

Isolation and identification of Gram negative psychrotrophic bacteria. Ten millilitres of each sample were diluted with peptone water and appropriate dilutions plated on plate count agar. The plates were incubated at 7 °C for 10 days for psychrotrophic counts (Guireaud, 1998).

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Bacterial isolates were purified by three successive transfers on plate count agar. Two hundred and fifty-eight (258) strains were characterised phenotypically (i.e. Gram stain and cell morphology). The following characteristics were determined for all Gram negative strains (181): mobility, catalase and cytochrome C oxydase production, oxidation/fermentative of glucose in Hugh-Leifson medium added of 0.1% glucose, acid and gas production from glucose, and H_2S production, as described by Richard (1983) and Guiraud (1998). Only oxydase positive and glucose fermentation negative strains were identified by using API 20NE galleries (Biomerieux, France).

An overnight culture in nutritive broth of each identified strain was used for studies of enzymatic activities. Plates were incubated at 25 ºC.

Production of extra-cellular lipases. The production of extracellular lipases was determined from diffusion agar assay on plate count agar plates that contained 0.1% Tween 20, 0.1% Tween 80 or 0.1% tributyrin. The plates were incubated at 25 °C for 72 h.

Lipolytic activity was shown by precipitated zone around and under each colony (Frank, 1997). Butter fat agar containing Victoria blue was used, and the enzymatic production was detected by a clear zone (yellow) around the colony. The production of extra-cellular phospholipase (lecithinase) was determined on plate count agar supplemented with 10% egg yolk emulsion as described by Dogan and Boor (2003); the appearance of an opaque ring, surrounding lecithinase-positive colonies, was recorded after 3 days of incubation at 25 °C. Each lipolytic activity was made in triplicate, and related to the area average (mm^2) of the zone displayed. The most active strains were then selected.

Lipolytic activities were measured, using API ZYM galleries (BioMerieux) only for selected strains.

Application of an experimental design for optimising esterase activity culture conditions.

Experimental design. Experimental matrix 2^{7-4} (De Meo *et al*., 1985) with seven factors, two levels (+1 and -1), and eight experiences were used to study the effects of the main environmental and nutritional factors on the esterase activity of each strain previously selected.

Inoculants cultures were grown on 100 ml of nutrient broth. Flasks were incubated for 24 h. Concentration of Tween 20, bacterial biomass, pH, temperature of incubation, agitation, phosphate potassium and lactose concentrations, were varied (Table 1).

The contrast was calculated by the formula:

$$L_i = \Sigma X_i Y_i/8$$

where L_i is the contrast, X_i is the sign of matrix (+ or -) and Y_i is the average of the two responses of each experience. L_i will be compared to 2 x E (E is the effect difference type) to estimate if the factor affected or not the response:

$$E = \sqrt{\frac{(\Sigma V_i / 8)}{16}}$$

where *Vi* is variance obtained from two responses of each experience.

If $| L_i | < 2 \times E \rightarrow$ the factor hasn't influenced the response.

If $| L_i | > 2 \times E \rightarrow$ the factor has influenced the response positively or negatively.

The eight experiences were made in duplicate (A and B) for each selected strain.

Esterase assay. Extracellular esterase activity was quantified using α-naphtyl acetate (Sigma, France) as the substrate according to the method described by Dupuis and Boyaval (1993) modified as follows: 200 µl substrate (Na derivatives 0.66 $mmol.l^{-1}$, dissolved in acetone (5% v/v) in sodium phosphate buffer (0.05 mol l^{-1}, pH 7.0) were added to 200 µl of the enzyme solution, enzymatic reaction occurred at 30 °C for 30 and 60 min. The reaction was stopped by the addition of 200 µl of 'Zym A' [TRIS-hydroxymethyl-aminoethane 25% (w/v), 12.5 N hydrochloric acid 11% (v/v) and sodium lauryl sulphate (SDS) 10% (w/v)]. Then the reaction was revealed with 200 µl of 'ZYM B' (Fast Blue BB (Sigma) 0.35% (w/v) in 2-methoxy-ethanol (Biomerieux). Absorbance was immediately determined at

TABLE 1 - Experimental matrix, with 7 parameters, 8 experiences at 2 levels, used for measuring extra-cellular esterase activity (ΔOD/min/ml) of the psychrotrophic strains isolated from refrigerated raw milk

Factors	Tween 20 (%)	Biomass (UFC ml^{-1})	pH	Temperature (°C)	KH_2PO_4/K_2HPO_4 (1/1) (%)	Lactose (%)	Agitation
Levels							
-	0.1	10^2	4	20	0.01	0.01	without
+	1	10^7	7	37	0.5	0.5	with
Experiences							
1	-	-	-	+	+	+	-
2	+	-	-	-	-	+	+
3	-	+	-	-	+	-	+
4	+	+	-	+	-	-	-
5	-	-	+	+	-	-	+
6	+	-	+	-	+	-	-
7	-	+	+	-	-	+	-
8	+	+	+	+	+	+	+
Contrast (L_i)	L1	L2	L3	L4	L5	L6	L7

-: low level, +: high level.

TABLE 2 - Lipolytic activitiy of *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Pseudomonas putida*, *Aeromonas hydrophila* and *Chryseomonas luteola* using agar diffusion method

	Isolates	Nm + Tween 20	Nm + Tween 80	Nm + egg yolk emulsion	Nm + butter victoria blue	Nm + tributyrin	Addition
P. fluorescens	48	+	+	+	+	-	4+
	61	+	+	-	+	+	4+
	75	+	+	+	+++	+	7+
	76	+	+	+	-	-	3+
	87	++	+	+	-	-	4+
	88	+	+	+	+	-	4+
	111	++	+	+	+++	-	7+
	126	+	+	+	+	+	5+
	128	++	+	+	+	-	5+
	133	++	+	+	+	-	5+
	149	+++	+	+	+	-	6+
	166	+	++	+	+	-	5+
	170	++	++	+	+	-	6+
	176	++	+	+	-	-	4+
	184	+	++	+	+	+	6+
	196	++	++	+	+	-	
	214	+++	++	+	+++	-	10+
	244	+	++	+	+	-	5+
P. cepacia	45	++	+	+	+	-	5+
	51	+	+	+	-	-	3+
	61	+	+	+	-	-	3+
	116	+++	++	+	+++	-	9+
	118	++	+	+	-	-	3+
	200	+	+	+	-	-	3+
	207	++	+	+	+++	-	7+
	211	+	+	++	-	-	4+
	215	++	+	+	+++	-	7+
	219	++	+	+	+++	-	7+
P. putida	117	++	+	+++	+++	-	9+
	127	++	+	+	++	-	6+
	144	+	+	+	+++	-	6+
	145	+	+	++	+	-	5+
	147	++	+	+	+	-	5+
	205	++	+	+	-	-	4+
A. hydrophila	46	++	+	+	+++	-	7+
	63	+	+	+	-	-	3+
	66	++	++	+	+	-	6+
	110	++	+	+	+	+	6+
	113	+	+	++	-	-	4+
	134	+	+	++	-	-	4+
	174	+	+	+	+++	-	6+
	181	++	+	+	+++	-	7+
	188	+	+	+	+	-	4+
	202	+	+	+	+++	-	6+
	203	+++	+	+	+++	-	8+
	239	+	+	+	+++	+	7+
	246	++	++	++	+++	-	9+
C. luteola	106	+	+	+	+	+	5+
	236	++	+	+	+	-	5+
	243	+	+	+	+++	-	6+
	250	+++	++	+	+++	-	9+
	251	++	+	+	++	-	6+

Nm: Nutrient medium. +: < 3 mm^2, ++: < 6 mm^2, +++ > 6 mm^2.

540 nm by a spectrophotometer (Jenway 6350). A unit (U) of enzyme specific activity was expressed as the change of 0.1 unit absorbance per min for 1 ml of culture.

Principal Components Analysis (PCA) of factors. The data obtained in matrix were used to make PCA of the seven environmental and nutritional factors for selected strains. It is a mathematical procedure that resolves multivariate data (contrasts values for each strain and each factor) into orthogonal components whose linear combination approximates the original data. These orthogonal components, the new variables, are called loadings, and permit to have more information about factors affected esterasic activities of strains.

RESULTS AND DISCUSSION

Identification of psychrotrophic Gram negative bacteria

Among the 258 strains of psychrotrophic bacteria, 83 Gram negatives, oxydase positive, glucose fermentation negative strains were identified using API 20NE galleries. Predominant genera were *Pseudomonas* (42%) and *Aeromonas* (16%) that are normally associated with raw milk. Five species were predominantly present: *Pseudomonas fluorescens* (20%), *Aeromonas hydrophila* (16%), *Pseudomonas cepacia* (13%), *Pseudomonas putida* (6%) and *Chryseomonas luteola* (5%).

Lipolytic activities from psychrotrophic bacteria

Agar diffusion assay

The five predominant species tested on different agar medium are illustrated in Table 2. The strains selected were *P. fluorescens* (214), *P. cepacia* (116), *P. putida* (117), *Aeromonas hydrophila* (246) and *C. lutelola* (250) which present the highest lipolytic activity on agar medium supplemented with different source of fat.

Lipolytic activities measured using API ZYM kit

The above selected strains were tested for their lipolytic activities using API ZYM system (Table 3). Esterase, esterase lipase and lipase enzymes were chosen to represent lipolytic activities of the five strains selected. In fact, lipases and esterases of all kinds are part of the serine hydrolase family (Tombs, 1995). The mode of action of these enzymes on triglycerides is to release fatty acids and glycerol. The free fatty acids possess undesirable flavour characteristics, which are imparted into the milk and milk products (Allen, 1994).

TABLE 3 - Psychrotrophic strains isolates exhibiting lipolytic activities measured using API ZYM kit (nanomoles of substrate hydrolysed)

Isolate	Lipolytic activity		
	Esterase (C_4)	Esterase lipase (C_8)	Lipase (C_{14})
P. fluorescens	10	40	10
P. cepacia	30	30	20
P. putida	20	30	5
A. hydrophila	20	20	5
C. luteola	20	30	5

The Gram negative strains tested in this study showed high esterase activity ranging between 20 nmol and 40 nmol. Nonetheless, *P. putida*, *A. hydrophila* and *C. luteola* had low lipase activity of 5 nmol, *P. fluorescens* and *P. cepacia* had 10 nmol and 20 nmol, respectively. The obtained results were in agreement with previous investigation (Mankaï *et al*., 2005) reporting that negative psychrotrophic strains had esterase and esterase lipase activi-

TABLE 4 - Contrasts of experimental matrix used to optimise esterase activity of *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Pseudomonas putida*, *Aeromonas hydrophila* and *Chryseomonas luteola*

Factors	Levels		Contrasts (L_i) calculated for the experimental matrix applicated for selected strains				
			P. fluorescens	*P. cepacia*	*P. putida*	*A. hydrophila*	*C. luteola*
Tween 20 (g/l)	+	10					
	–	1	–0.0349	–0.0707	–0.0265	–0.052	–0.047
Biomass (CFU/ml)	+	10^7					
	–	10^2	0.0396	0.0484	0.0114	0.0104	0.018
pH	+	7					
	–	4	0.0226	–0.0426	0.0418	0.0874	0.034
Temperature (°C)	+	37					
	–	18	0.0064	–0.0065	–0.0004	0.0594	0.019
Potassium phosphate (g/l)	+	5					
	–	0.1	–0.0085	–0.0361	0.0419	–0.0021	0.0635
Lactose (g/l)	+	5					
	–	0.1	0.0138	–0.0064	0.0584	0.0621	0.0635
Agitation	+	with					
	–	without	0.0192	0.03467	0.0251	0.0191	0.034
2 x effect difference			0.009	0.03	0.006	0.027	0.02

ty of 20-40 nmol hydrolysed substrate, with a quasi absence of lipase activity. The production of esterase and esterase lipase by the respective psychrotrophic organisms suggested that the fat component in milk would be subjected to pre-manufacture lipolysis and possibly to post-manufacture lipolysis. Hence, even if the populations of such producing organisms are minimised or eliminated during processing, the enzymes may survive heat treatment and cause lipolysis (post-manufacture lipolysis) (Aaku, 2004).

Optimisation of esterase activity culture conditions

The effect of different conditions (Tween 20 concentration, bacterial biomass, pH, temperature of incubation, agitation, phosphate potassium and lactose concentration) on extra-cellular esterase activity of the five strains previously selected by using experimental matrix is illustrated in Table 4.

Tween 20, added to medium broth, affected esterase activity in each strain tested negatively. On the other hand, these strains had a great affinity with this substrate when they were tested with the agar diffusion method. The apparent decrease in enzyme activity in presence of Tween 20 could be due to the absorption of the hydrophobic substrate, α-naphtyl acetate, and to the fat of Tween 20 as was reported by Griffiths (1989).

Biomass concentration increased esterase activity for all strains tested (*P. fluorescens*, *P. cepacia*, *P. putida*, *A. hydrophila*, and *C. luteola*). The results obtained by Fairbairn and Law (1986) were close to ours. Indeed, the above mentioned researchers pointed out that the maximum enzymatic activity was reached for a bacterial concentration of 10^7 CFU/ml of psychrotrophs. This bacterial count reached about 10^7 CFU/ml and affected the organoleptic quality of milk, even though this milk had been submitted heat treatment (Dogan and Boor, 2003).

In most cases (*P. fluorescens*, *P. putida*, *A. hydrophila*, and *C. luteola*), an important esterasic activity was obtained at pH = 7. Griffiths (1989) found that lipases was synthesised when reaching pH ≥ 7. In addition, enantioselective esterase from *Pseudomonas fluorescens* KCTC 1767 showed maximum activity at pH 9-10 (Ji-Heui *et al*., 2004). Hence, pH around 7 is optimal for esterase of selected strains. In fact reaction speed decreased when H^+ concentration (pH) changed in the medium, because the tri-dimensional structure of protein is modified (Tortora *et al*., 2003).

Pseudomonas can grow at 37 ºC (Ternström *et al*., 1993), more over; Al-Saleh and Zahran (1999) have found that this temperature is optimum for the growth of *Pseudomonas fluorescens* RM4. However, Buchon *et al*. (2000) indicate that the optimal growth temperature of some psychrotrophic bacteria is situated between 15-20 ºC.

On the other hand, either at 18 or 37 °C incubation temperature, esterasic activities are similar. In fact, temperature was shown to be an insignificant factor in the matrix plan. The results of the present study do not seem to concur with those of Buchon *et al*. (2000) who have reported that temperature depended significantly on the amount of production of extra-cellular enzymes (cellulase, pectate lyase, chitinase activities,…) by psychrotrophic bacteria. Moreover, it was shown that the activity of proteolytic enzymes increased with temperature (Mankaï *et al*., 2005). However, Gügi *et al*. (1991) have suggested that 17.5 ºC is the optimal temperature for the extra-cellular protease of *Pseudomonas fluorescens* MF0.

Laurent *et al*. (2000) investigated production pectate lyases and cellulases by *Chryseomonas luteola* strain MFCL0. They suggested that *Chryseomonas luteola* MFCL0 cultures were grown at different temperatures (8, 11, 14, 17, 20, 24 and 28 ºC) and in different media at pH 7. They have suggested that regulated genes of these enzymes are also complex by temperature that depends on the composition of the medium.

Concentration of 5 g/l phosphate potassium in the medium increased esterasic activities of *A. hydrophila*, *C. luteola,* while that of *P. cepacia* decreased. Moreover, phosphate potassium concentration did not affect esterasic activities of *P. fluorescens* and *P. putida*. These fluctuating results can be due to an interaction between Tween 20 and pH, and not to a real effect of phosphate potassium.

Lactose was assessed as supplement to the medium exerting positive effect on esterasic activities of the most psychrotrophic strains studied. Lactose is a carbon source which is used by bacteria. Thus, it can be concluded that additional carbon source in the medium stimulates esterase production, as reported by Raja Noor Zaliha *et al*. (2005). They have also reported that medium containing sorbitol as carbon source increased protease production of *Pseudomonas aeruginosa*, compared to basal medium.

Agitation has increased the esterasic activities of *Pseudomonas* and *Aeromonas* strains. In fact, these germs are obligate aerobes; so continuous aeration is generally required for their optimal growth and for enzyme production (Malik *et al*., 1985; Griffiths, 1989).

Evaluation of the factors by PCA

A PCA was carried out on seven environmental and nutritional factors for strains previously isolated. The similarity map defined by the first two principal components took into account 71.7% of the total variance. The first component (PC1) by itself condensed 47.8% and the second component (PC2) represented 23.9% of the total information. Loading coefficients obtained from the application of PCA to the data are shown in Table 5.

TABLE 5 - Factor loadings of principal components of seven environmental and nutritional factors

Factor	Component	
	PC1 (47.8%)	PC2 (23.9%)
Tween 20 concentration (L1)	-0.359	-0.283
Biomass level (L2)	0.922 *	0.017
pH (L3)	-0.823 *	0.436
Temperature (L4)	-0.684 *	0.531
Potassium phosphate concentration (L5)	-0.599	-0.757 *
Lactose concentration (L6)	-0.887 *	-0.373
Agitation (L7)	0.282	-0.641 *

*: high correlation between environmental and nutritional factors and principal components (Factor loadings ≥ at 0.7)

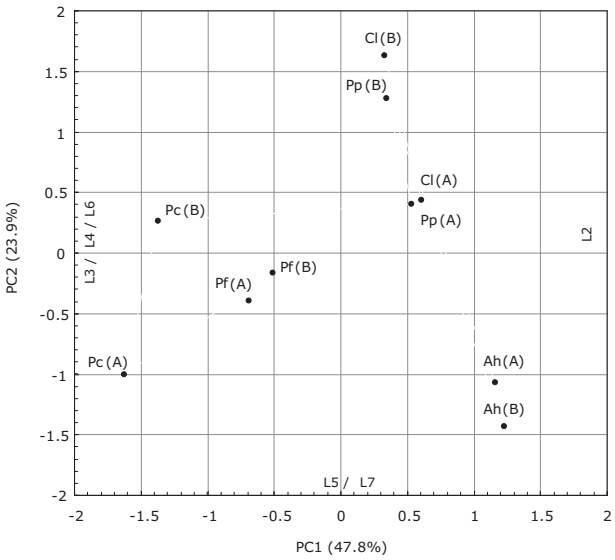

FIG. 1 - A PCA-scores plot of Gram negative strains comportment. *Pseudomonas fluorescens* (*Pf*), *Pseudomonas cepacia* (*Pc*), *Pseudomonas putida* (*Pp*) and *Aeromonas hydrophila* (*Ah*), and *Chryseomonas luteola* (*Cl*). Environmental and nutritional factors: biomass level (L2), pH (L3), temperature (L4), potassium phosphate concentration (L5), lactose concentration (L6) and agitation (L7).

PC1 has shown high negative correlation with pH (L3), temperature (L4) and lactose concentration (L6). High positive correlation was found between PC1 and biomass level (L2). PC2 has shown high negative correlation with potassium phosphate concentration (L5) and agitation (L7). A PCA-scores plot of strains comportment is plotted in Fig. 1 for the two first PC.

Regarding PC1, *Pseudomonas fluorescens* and *Pseudomonas cepacia*, presented negative score values, whereas the other strains exhibited positive values. Strains showed differences in factors having an influence on their esterasic activities. However, *Pseudomonas putida* and *Aeromonas hydrophila* were similarly influenced, especially by potassium phosphate concentration.

Changes in esterasic activity of different strains were mostly explained by PC1. Then, activities of *Pseudomonas fluorescens* and *Pseudomonas cepacia* were explained by low Biomass level (L2) and by high pH (L3), temperature (L4) and lactose concentration (L6). However, esterasic activities of *Aeromonas hydrophila* were explained by high Biomass level (L2) and by low pH (L3), temperature (L4) and lactose concentration (L6).

The strains *Pseudomonas putida*, *Chryseomonas luteola* and *Aeromonas hydrophila* were differentiated by potassium phosphate concentration (L5) and agitation (L7). The esterasic activities of *Pseudomonas putida*, *Chryseomonas luteola* were explained by low potassium phosphate concentration and agitation, and *Aeromonas hydrophila* was explained by high potassium phosphate concentration and agitation.

These results completed those obtained in Table 3. In fact, matrix study indicated that biomass level and agitation concentration affect the esterase activity of each strain. Thus, PCA procedure demonstrates that *Aeromonas hydrophila*, *Pseudomonas putida* and *Chryseomonas luteola* are mostly strains affected by these factors. On the other hand, lactose concentration increased not only esterase activities of *Aeromonas hydrophila* (Table 3) but also those of *Pseudomonas putida* (Fig. 1).

From Fig. 1, we can observe 3 groups: (i) *Pseudomonas fluorescens* and *Pseudomonas cepacia,* (ii) *Pseudomonas putida* and *Chryseomonas luteola,* (iii) *Aeromonas hydrophila*.

CONCLUSION

Pseudomonads and *Aeromonas* are major genera of spoilage bacteria in refrigerated raw milk. The production of lipolytic enzymes by predominant contaminating gram negative microorganisms was high and will also lead to short shelf life of the product. Esterasic activity increases at 10^7 CFU/ml, pH = 7, with permanent agitation and 0,5% of lactose concentration. However, the addition of 10% Tween 20 decreased esterasic activity. PCA permits to know that esterasic activity of some strains are more affected by some factors than others. Lastly, dairy industries need to put at a disadvantage factors which increased lipolytic and esterasic activities. This precaution would ameliorate the organoleptic quality of dairy products.

REFERENCES

Aaku E.N., Collison E.K., Gashe B. A., Mpuchane S. (2004). Microbiological quality of milk from two processing plants in Gaborone Botswana. Food Control, 15: 181-186.

Allen J.C. (1994). Rancidity in dairy products. In: Allen J.C., Hamilton R.J., Eds, Rancidity in Foods, 3rd edn., Blackie Academic and Professionnal, London, pp. 179-762.

Al-Saleh A.A., Zahran A.S. (1999). Synthesis of extracellular lipase by a strain of *Pseudomonas fluorescens* isolated from raw camel milk. Food Microbiology, 16: 149-156.

Braun P., Fehlhaber K., Klug C., Kopp K. (1999). Investigations into the activity of enzymes produced by spoilage-causing bacteria: a possible basis for improved shelf-life estimation. Food Microbiology, 16: 531-540.

Buchon L., Laurent P., Gounot A.M., Guespin-Michel J.F. (2000). Temperature dependence of extracellular enzymes production by psychrotrophic and psychrophilic bacteria. Biotech. Lett., 22: 1577-1581.

Deeth H.C. (2002). Lipolysis. In: Roginsky H., Fuquay J.W., Fox P.F., Eds, Encyclopedia of Dairy Sciences, vol. 1, Academic Press, New York, pp. 1595-1601.

De Meo M. Larget M., Phan-Than-Luu R., Mathieu D., Duménil G. (1985). Application des plans d'expériences à l'optimisation des milieux et des conditions de cultures en fermentation. Rev. Biol. Sci., 4: 45-49.

Dogan B., Boor K.J. (2003). Genetic diversity and spoilage potentials among *Pseudomonas* spp. Isolated from fluid milk products and dairy processing plants. Appl. Environ. Microbiol., 69: 130-138.

Dousset X., Pinet X., Guillaumin B., Janvier P. (1986). Evolution de la qualité bactériologique du lait au cours de la collecte, Lait, 66: 75-87.

Dousset X., Demaimay M., Ravaud C., Levesque A., Pinet X., Kergo Y. (1988). Influence de la température de réfrigération du lait sur la protéolyse et l'amertume du lait UHT au cours de son stockage. Lait, 68: 143-156.

Dupuis C., Boyaval P. (1993). Esterase activity of dairy *Propionibacterium*. Lait, 73: 345-356.

Fairbairn D.J., Law B.A. (1986). Proteinases of psychrotrophic bacteria. Their origin, production, properties, effects and control. J. Dairy Res., 53: 139-177.

Frank J.F. (1997). Milk and dairy products. In: Doyle M.P., Beuchat L.R., Montville T.J., Ed., Food Microbiology, Fundamentals and Frontiers, American Society of Microbiology, Washington, D.C., pp. 101-116, 581-594.

Griffiths M.W. (1989). Effect of temperature and milk fat on extracellular enzyme synthesis by psychrotrophic bacteria during growth in milk. Milchwissenschaft, 44: 537-604.

Gügi B., Orange N., Hellio F., Burini J. F., Guillou C., Leriche F., Guespin-Michel J.F. (1991). Effect of growth temperature on several exported enzyme activities in the psychrotrophic bacterium *Pseudomonas fluorescens*. J. Bacteriol., 12: 3814-3820.

Guiraud J.P. (1998). Microbiologie Alimentaire. Dunod, Paris.

Ji-Heui K., Gi-Sub C., Seung-Bum K, Won-Ho K., Jin-Young L., Yeon-Woo R., Geun-Joong K. (2004). Enhanced thermostability and tolerance of high substrate concentration of an esterase by directed evolution. J. Mol. Catal., .27: 169-175.

Laurent P., Buchon L., Guespin-Michel J., Orange N. (2000). Production of pectate lyases and cellulases by *Chryseomonas luteola* MFCL0 depends on growth temperature and the nature of the culture medium: evidence for two critical temperatures. Appl. Environ. Microbiol., 66: 1538-1543.

Malik R.K., Prasad R., Mathur D.K. (1985). Effect of some nutritional and environmental factors on extracellular protease production by *Pseudomonas* SP. B25. Lait, 65: 169-183.

Mankaï M., Ben Moussa O., Hassouna M. (2005). Influence des conditions de culture sur l'activité protéolytique extracellulaire de souches psychrotrophes isolées à partir de lait cru tunisien réfrigéré - Leur caractérisation enzymatique par le système API ZYM. Ind. Alim. Agric., 122 (4): 7-13.

MC Phee J.D., Griffiths M.W. (2002). Psychrotrophic bacteria, *Pseudomonas* spp. In: Roginsky H., Fuquay J.W., Fox P.F., Eds, Encyclopedia of Dairy Sciences, Vol 4, Academic Press, New York, pp. 2340-2351.

Miranda G., Gripon J.C. (1986). Origine, nature et incidences technologiques de la protéolyse dans le lait. Lait, 66: 1-18.

Rahman R.N.Z.R.A., Geok L.P., Basri M., Salleh A.B. (2005). An organic solvent-tolerant from *Pseudomonas aeruginosa* strain K. Nutritional factors affecting protease production. Enzyme Microb.Tech., 36: 749-757.

Richard J. (1983). Nature de la flore microbienne dominante et sous-dominante des laits crus très pollués. Lait, 63, 148-167.

Shah N.P. (1994). Psychrotrophs in milk. Milchwissenschaft, 49: 432-437.

Ternström A., Lindberg A.M., Molin G. (1993). Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus*. J. Appl. Bacteriol., 75: 25-34.

Tombs M.P. (1995). Enzymes in the processing of fats and oils. In: Tucker G.A., Woods L.F.J., Eds, Enzymes in Food Processing, 2nd edn., Blackie Academic and Professionnal, London, pp. 268-281.

Tortora G.J., Funke B.R., Case C.L. (2003). Le métabolisme microbien. In: Tortora G.J., Funke B.R., Case C.L., Eds, Introduction à la Microbiologie, 7th edn., Renouveau Pédagogiques Inc. Edition, Canada, pp. 124-168.