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Original article

Acceleration of blue cheese ripening by cheese slurry and extracellular enzymes of Penicillium roqueforti

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Summary — An attempt has been made to accelerate flavour development of blue cheese. Ripened cheese slurry has been incorporated into blue cheese curd at a rate of 1 and 2% respectively. A mixture of proteinases and lipases (1:1) of *P. roqueforti* has been incorporated into blue cheese curd at the proportions of 0.01 and 0.02 respectively. Blue cheese has also been made without additives (control).

Addition of either cheese slurry or enzyme mixtures to blue cheese curd has accelerated the development of the characteristic flavour of blue cheese. These additives have stimulated the formation of soluble nitrogenous compounds, free amino acids, volatile fatty acids and total carbonyl compounds.

Blue cheeses containing 2% ripened slurry or 0.02% *P. roqueforti* enzymes have higher organoleptic properties than the control after 45 d of ripening. The concentrations of the previously mentioned compounds in the same period of ripening are higher than those found in control cheese after 60 d.

ripening — blue cheese — protease — lipase — *Penicillium roqueforti* — proteolysis — lipolysis — flavour

Résumé — Accélération de l'affinage du fromage bleu par du «caillé hydraté» homogénéisé et par des enzymes extracellulaires de *Penicillium roqueforti*. *Un essai a été fait pour accélérer le développement de la flaveur du fromage bleu. Du «caillé hydraté» homogénéisé a été incorporé dans du caillé de fromage bleu à raison de 1 à 2%.*

Un mélange de protéases et de lipases (1:1) de P. roqueforti *a été également incorporé dans du caillé de fromage bleu à raison de 0.01 et 0.02%. Le fromage bleu a été aussi fabriqué sans additifs (témoin).*

L'addition soit du «caillé fluide» homogénéisé, soit du mélange d'enzymes, a accéléré le développement des flaveurs caractéristiques.

Ces addifits ont stimulé la formation de composés azotés solubles, d'acides aminés libres, d'acides gras volatils et des composés carbonylés totaux au cours de l'affinage.

Les fromages bleus contenant soit 2% de «caillé fluide» homogénéisé soit 0,02% d'enzymes de P. roqueforti *possèdent de meilleures propriétés organoleptiques que le témoin après 45 jours de maturation. Les concentrations des composés antérieurement mentionnés sont plus importantes dans la même période d'affinage que celles trouvées dans le témoin après 60 jours.*

affinage — fromage bleu — protéase — lipase* — Penicillium roqueforti — *protéolyse — lipolyse — arôme

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INTRODUCTION

Cheese ripening is a complex process which involves several biochemical reactions leading to the fermentation of lactose, breakdown of casein and hydrolysis of milk fat (Law, 1984).

Blue cheese undergoes extensive proteolysis during ripening (Kinsella and Hwang, 1976a; Hewedi and Fox, 1984) and the free fatty acids and methyl ketones produced are major contributors to the characteristic flavour of blue cheese (Lawrence, 1966; Jolly and Kosikowski, 1975; Kinsella and Hwang, 1976a,b; King and Clegg, 1979).

Several methods have been proposed to improve the quality of different types of cheese and shorten their ripening periods, such as increasing the ripening temperature, addition of modified starter (heat shocked), cheese slurry, trace elements or addition of enzymatic preparations (Hofi *et al.*, 1973; Abdel Salam *et al.*, 1981; Abdel Baky *et al.*, 1982, 1986). Jolly and Kosikowski (1975) accelerated the development of blue cheese flavour by adding microbial lipases.

The incorporation into an aseptic curd of aspartyl protease of *Penicillium roqueforti* or metalloprotease of *P. camemberti* resulted in formation of high and low molecular weight peptides without liberation of amino acids. The liberation of amino acids resulted from the action of exopeptidases (Gripon *et al.*, 1977).

Recently, the Polish National Committee of the IDF (1987) concluded that the most suitable method of accelerating cheese ripening is the use of enzymatic preparations obtained from the natural microflora of cheeses.

Therefore, the present investigation was carried out to accelerate blue cheese ripening, using either ripened blue cheese slurry or a mixture of crude proteolytic and lipolytic enzymes (1:1) secreted by *P. roqueforti*.

MATERIALS AND METHODS

Materials

Fresh cow's milk of 3.5% fat and 9% S.N.F. was used in this investigation. A rennet powder (1:100.000) was obtained from L.C. Glad Company A/S, Copenhagen, Denmark.

Pure cultures of *S. lactis* and *S. diacetylactis* were obtained from Hansen's Laboratory. The cultures were activated before being used. *P. roqueforti* strain R64A was kindly supplied by Dr Lenoir (INRA, Laboratoire de Recherche de la Chaire de Technologie, France).

Preparation of cheese slurry

The methods described by King and Clegg (1979) for preparing aseptic curd slurry were adopted.

Cow's milk of 3.5% fat and 9% S.N.F. was heated to 71—72°C for 15 s, rapidly cooled to a setting temperature of 35°C and acidified to pH 5.6 with lactic acid. Rennet was then added to coagulate the milk (40 min). The coagulum was cut and then it was ladled in cheese clothes for complete drainage over 24 h. The resultant curd was firstly mixed with 4% NaCl, and transfered in to 500 ml conical flasks. The curd was sterilized at 120°C for 15 min. Sterilized curd was aseptically inoculated with 0.5% of *Penicillium roqueforti* mycelium and incubated at 25°C for 15 days.

Production of crude enzyme preparations

P. roqueforti strain R64A was cultured at 25°C for 5 days in Czapek-trypticase modified

medium (Lenoir *et al.*, 1979). The pH of the medium was adjusted to 4.0 for the production of aspartyl protease and acid carboxypeptidase, 6.5 for the production of metalloprotease, aminopeptidase and neutral carboxypeptidase, and 7.5 for the production of lipase (Lamberet and Lenoir, 1972).

The media were inoculated with *P. roqueforti* strain R64A. At the end of the incubation period, the vegetative growth was removed by centrifugation followed by filtering. The cell free extracts containing the enzymes were centrifuged at 2000 *g* for 20 min. Equal volumes of extracts containing proteolytic enzymes were mixed and the enzymes were precipitated by acetone at a concentration of 80%. The mixture was immediately centrifuged (20 min x 2000 g) and the supernatant was discarded. The same procedure was followed with precipitation of lipolytic enzymes. The precipitate resulting from the extracts containing either proteolytic enzymes or lipolytic enzymes was weighed and dissolved in 0.025 M Na-citrate buffer pH 4.0. Insoluble material was removed by centrifugation (30 min x 6000 g). The mixture of crude proteolytic and lipolytic enzymes (1:1) was incorporated into the blue cheese curd at a level of 0.01 and 0.02%.

Cheesemaking

Blue cheese was made from cow's milk containing 3.5% fat. Cow's milk was pasteurized at 71—72°C for 15 s, and cooled to 30°C. A starter culture consisting of *Streptococcus lactis* and *Streptococcus diacetylactis* (1:1) at a rate of 2% was added to cheese milk. Cheese making was completed as described by Kosikowski (1977). The resultant curd was inoculated with *P. roqueforti* and divided into 5 equal parts. The first part was left without additives and served as a control. Ripened cheese slurry was incorporated into cheese curd of the second and the third parts at a rate of 1 and 2% respectively. A mixture of crude proteolytic and lipolytic enzymes of *P. roqueforti* (1:1) was added at a rate of 0.01 and 0.02% of curd weight to the fourth part of the curd respectively. Cheese was ripened at 8—10°C and 95% RH for 60 days.

Organoleptic properties

Cheese samples were organoleptically evaluated by the method of Spreer (1978) with maximum score points of 4.5, 3.5., 2.0 and 10.0 for external appearance, internal appearance, odour and taste respectively.

Chemical analysis

Cheese samples were analysed after salting, then after 30, 45 and 60 days, for moisture, fat, salt, total nitrogen, soluble nitrogen and pH as described by Ling (1963). Amino acid nitrogen was determined according to the method of Stadhouders (1959).

Total volatile fatty acids

Free volatile fatty acids were determined by the method of Kosikowski (1977).

Tyrosine and tryptophane

Tyrosine and tryptophane were spectrophotometrically determined by the method described by Vakaleris and Price (1959).

Total carbonyl compounds

The total carbonyl concentration of blue cheese was determined by preparing 2,4-dinitrophenyl hydrazones of carbonyl compounds by the method of Lawrence (1965) and measuring the optical density at 340 nm, using Safas Monaco Type 210 Spectrophotometer. Total carbonyl content was quantified using the molar extinction coefficient of 22,500, since methyl ketones accounted for more than 90% of total carbonyls in solution.

Statistical analysis

The effects on scores of blue cheese of the addition of both ripened cheese slurry and

Table I. Chemical composition of ripened blue cheese slurry.

Components	*Values*
Moisture %	55.75
Fat %	20.20
Salt %	3.90
Total N %	3.55
Soluble N *	35.80
Tyrosine **	150.40
Tryptophan **	120.70
T.V.F.A. ***	40.20
pH	5.80

* Values are expressed as % of total nitrogen.
** Values are expressed as mg/100 g of cheese.
*** T.V.F.A. : Total volatile fatty acids are expressed as ml of 0.01 N NaOH/100 g of cheese.

proteolytic and lipolytic enzymes to blue cheese curd were statistically analysed by the F test. In cases of significant F, differences between treatments were examined by Duncan's multiple range test (Snedecor and Cochran, 1957).

RESULTS AND DISCUSSION

Cheese slurry

Table I shows the chemical composition of ripened blue cheese slurry. Ripened blue cheese slurry contained higher levels of soluble nitrogenous compounds, free amino acids and carbonyl compounds after 15 days of incubation. The flavour of typical blue cheese has developed during this period. Farag (1987) found that inoculation of aseptic blue cheese curd with different *P. roqueforti* strains showed a typical blue cheese flavour after 2 weeks of incubation at 25°C.

Gross chemical composition

Table II shows that either addition of ripened cheese slurry or a mixture of proteolytic and lipolytic enzymes (1:1) to cheese curd had a slight effect on the moisture, fat and salt contents of blue cheese after salting and during ripening. Blue cheese containing either ripened slurry or enzymes mixture had different pH values compared with the control. The changes in the pH values between the control cheese and experimental cheeses were small after salting. As ripening proceeded, cheese containing either ripened slurry or enzymes mixture showed higher pH values than the control. On the other hand, cheese made with ripened slurry had slightly higher pH values than those made with enzyme mixtures after 45 days of cheese ripening. This may be due to the different rate of casein degradation at this stage of cheese ripening (Table III).

Generally, the pH values of both control cheese and all experimental cheeses gradually increased but at different rates during ripening. The observed difference between the control cheese and cheese containing slurry or enzyme mixtures may be due to the different rate of proteolysis occuring in blue cheeses containing the above mentioned additives (Table III). Similar results were obtained by Farag (1987).

The general trend of chemical composition of blue cheese containing ripened slurry agreed with that observed by Abdel Baky *et al.* (1982). They found that the utilization of cheese slurry to accelerate

Table II. Gross chemical composition of blue cheese as affected by cheese slurry and enzymes mixture of *P. roqueforti* *.
Composition chimique globale du fromage bleu influencée par le caillé hydraté et des mélanges d'enzymes de P. roqueforti *.

Components	*Ripening period (days)*	*Without additives (control)*	*Additives*			
			Cheese slurry		*Enzyme mixtures*	
			1%	*2%*	*0.01%*	*0.02%*
Moisture	F	48.7	48.8	48.4	48.8	48.8
	30	45.6	45.7	45.8	45.6	44.6
	45	44.5	44.6	44.7	44.4	44.6
	60	44.3	44.4	44.4	44.2	44.4
Fat (DM)	F	45.6	45.9	46.1	45.7	45.7
	30	45.4	45.8	46.0	45.6	45.6
	45	45.4	45.8	45.9	45.5	45.5
	60	45.4	45.8	45.8	45.6	45.4
Salt	F	3.6	3.7	3.8	3.7	3.8
	30	3.7	3.8	3.9	3.8	3.8
	45	3.8	3.9	3.9	3.9	3.9
	60	3.8	—	3.9	3.9	3.9
pH	F	4.8	4.9	4.9	4.9	4.9
	30	5.2	5.4	5.6	5.3	5.5
	45	5.8	6.2	6.4	5.9	6.14
	60	6.2	6.7	6.8	6.5	6.7

F = after salting. DM : dry matter. * = average of 3 determinations.

ripening of Egyptian hard cheese (Ras) has a slight effect on the gross chemical composition of cheese.

Ripening indices

Changes in soluble nitrogen, amino acid nitrogen, soluble tyrosine and tryptophan contents, free volatile fatty acids and total carbonyl compounds were taken as indices of testing blue cheese ripening.

Soluble nitrogen compounds and amino acids

Table III indicates that the addition of ripened blue cheese slurry or enzymes mixture of *P. roqueforti* to blue cheese curd stimulated the formation of soluble nitrogenous compounds, tyrosine and tryptophan. However, the levels of these compounds were higher in cheese containing ripened blue cheese slurry

Table III. Soluble nitrogen compounds and amino acids in blue cheese as affected by cheese slurry and enzymes mixture of *P. roqueforti* during ripening*.
Composés azotés solubles et acides aminés dans le fromage bleu influencés par le caillé hydraté et des mélanges d'enzymes de P. roqueforti *au cours de l'affinage **.

Components	*Ripening period (days)*	*Without additives (control)*	*Additives*			
			Cheese slurry		*Enzyme mixtures*	
			1%	*2%*	*0.01%*	*0.02%*
Soluble N **	F	9.14	9.84	10.65	9.24	10.20
	30	17.44	25.55	28.74	23.24	25.75
	45	28.64	38.90	40.90	35.74	37.90
	60	32.56	45.44	50.70	44.79	45.74
Amino N **	F	1.44	1.66	1.84	1.72	1.84
	30	5.60	7.40	7.94	6.94	7.50
	45	7.40	9.64	10.49	8.55	9.40
	60	9.77	11.84	12.50	11.26	12.25
Tyrosine ***	F	25.45	28.94	32.40	40.40	45.90
	30	150.40	187.20	240.60	230.55	260.50
	45	277.60	380.44	420.70	395.44	425.70
	60	302.42	442.74	490.40	455.60	485.50
Tryptophan ***	F	15.45	20.44	24.45	26.70	30.40
	30	95.65	115.55	140.60	125.88	155.50
	45	185.40	210.62	270.40	220.70	235.35
	60	210.74	250.44	295.36	240.44	279.40

F = after salting. * = average of 3 determinations. ** = % of total nitrogen. *** = mg/100 g of cheese.

than those containing enzyme mixtures or control blue cheese during ripening. The increased rate of both soluble nitrogenous compounds and amino acids was proportional to the amount of added cheese slurry and *P. roqueforti* enzyme mixtures. Addition of ripened blue cheese at a rate of 2% to the curd was more effective in this respect. Concentrations of soluble nitrogen, amino acid nitrogen and amino acids of blue cheese containing 2% of ripened slurry or 0.02% of *P. roqueforti* enzyme mixtures, at 45 days of cheese ripening, were higher than those of control at the end of the ripening period. The high rate of proteolysis in blue cheese containing ripened cheese slurry or mixture of proteolytic and lipolytic enzymes could be explained on the basis that cheese slurry contained *P. roqueforti*, which secretes a range of very active endo and exopeptidases which catalyse the degradation of casein (Zevaco *et al.*, 1973; Lenoir *et al.*, 1979).

On the other hand, cheese slurry contains a range of soluble, nitrogenous and amino acids compounds which probably stimulate the growth of *P. roqueforti* and enhance the formation of flavour compounds (Table I).

The results obtained agreed with those reported by Desmazeaud *et al.* (1976).

Table IV. Free volatile fatty acids of blue cheese as affected by cheese slurry and enzymes mixture of *P. roqueforti* * during ripening.
Acides gras volatils du fromage bleu influencés par le caillé hydraté et des mélanges d'enzymes de P. roqueforti * *au cours de l'affinage.*

Components	*Ripening period (days)*	*Without additives (control)*	*Additives*			
			Cheese slurry		*Enzyme mixtures*	
			1%	*2%*	*0.01%*	*0.02%*
T.V.F.A. **	F	2.4	3.2	4.1	3.8	4.5
	30	18.4	35.6	50.8	30.4	42.4
	45	36.2	55.7	74.4	60.4	65.2
	60	56.8	71.4	86.7	79.2	92.8
T.C.C. ***	F	25.40	32.45	35.40	34.40	36.70
	30	185.62	220.42	245.34	225.40	250.40
	45	245.70	450.74	759.40	412.87	750.37
	60	300.42	495.42	759.40	438.35	790.45

* = average of 3 determinations. ** T.V.F.A. = total volatile fatty acids are expressed as ml of 0.01 N NaOH/100 g of cheese. *** T.C.C. = total carbonyl compounds are expressed as μmol/100 g of cheese.

They found that the proteolysis is very intensive in cheese curd inoculated with a strain of *P. roqueforti.* At the end of ripening, the soluble nitrogen at pH 4.6, non-protein nitrogen and amino acid nitrogen, calculated as percent of total nitrogen, were 50, 30, and 10% respectively.

Free fatty acids and carbonyl compounds

Table IV shows the changes of free volatile fatty acids and carbonyl compounds in blue cheese containing ripened slurry or a mixture of proteolytic and lipolytic enzymes of *P. roqueforti.* Results showed that control blue cheese contained lower concentrations of free volatile fatty acids and carbonyl compounds than experimental cheeses made with either added cheese slurry or enzymes mixtures. Incorporation into cheese curd ripened slurry or enzyme mixtures accelerated the formation of free volatile fatty acids (C_2–C_{10}) and total carbonyl compounds. These results could be explained on the basis that cheese slurry and enzyme mixtures contain acid lipases which are responsible for the liberation of short chain fatty acids from triglycerides of milk fat. *Penicillium roqueforti* can produce at least 2 lipases that are distinguished by their optimum pH. Short-chain fatty acid specificities of these lipases influence the organoleptic qualities of blue veined cheese, because the amounts of each lipase vary widely from one cheese to another, being particularly related to the strain used and the ripening (Imamura and Kataoka, 1963). These volatile fatty acids are transformed in methyl ketones by either the spores or the mycelium of *P. roqueforti* (Dwivedi and Kinsella, 1974; Kinsella and Hwang, 1976b). Addition of ripened blue cheese slurry was more

Table V. Organoleptic properties * of blue cheese as affected by cheese slurry and enzymes mixture of *P. roqueforti.*
*Propriétés organoleptiques * du fromage bleu influencées par le caillé hydraté et les mélanges d'enzymes de* P. roqueforti.

Characteristics	*Ripening period (days)*	*Without additives (control)*	*Additives*			
			Cheese slurry		*Enzyme mixtures*	
			1%	*2%*	*0.01%*	*0.02%*
External appearance (4.5)		4.0	4.0	4.0	4.0	4.0
Internal appearance (3.5)		2.8	2.9	3.1	2.7	3.0
Odour (2.0)	30	1.0	1.4	1.6	1.3	1.5
Taste (10.0)		6.4	7.2	8.5	7.2	7.4
Total (20.0)		14.2 ± 0.4	15.5 ± 0.2	17.2 ± 0.3	15.2 ± 0.1	15.9 ± 0.3
External appearance (4.5)		3.9	3.9	3.8	3.8	3.8
Internal appearance (3.5)		2.9	3.1	3.2	3.0	3.0
Odour (2.0)	45	1.3	1.5	1.7	1.4	1.6
Taste (10.0)		7.6	8.1	8.3	8.1	8.2
Total (20.0)		15.7 ± 0.4	16.6 ± 0.2	17.0 ± 0.1	16.3 ± 0.4	16.6 ± 0.2
External appearance (4.5)		3.8	3.8	3.8	3.7	3.7
Internal appearance (3.5)		3.0	3.1	3.2	3.1	3.2
Odour (2.0)	60	1.5	1.7	1.9	1.6	1.7
Taste (10.0)		8.0	8.1	8.2	8.2	8.0
Total (20.0)		16.3 ± 0.3	16.7 ± 0.2	17.1 ± 0.2	16.6 ± 0.2	16.6 ± 0.2
Total general mean **		15.4 **	16.3 **	17.1 **	16.0 **	16.4 **

* Mean ± SEM of 3 determinations. ** Values in the same row differ significantly ($P > 0.05$).

effective in this respect. The figures obtained were higher than those found by Godinho and Fox (1981) and agreed with those obtained by King and Clegg (1979) and Farag (1987).

Organoleptic properties

Table V shows the effect of the addition of either ripened cheese slurry or mixture of proteolytic and lipolytic enzymes (1:1) of *P. roqueforti* on the organoleptic properties of blue cheese during ripening. Organoleptic examinations showed that both cheeses containing ripened slurry or enzyme mixtures gave good organoleptic qualities of blue cheese compared with the control, but between the different treatments and the control cheese there were significant differences. However, best quality cheese was obtained when the cheese contained 2% blue cheese slurry. Descending order of quality were obtained with cheese made with 0.02% enzymes mixture, cheese containing 1% slurry and cheese made with added 0.01% enzymes mixture when compared with the control (Table V). Cheese containing 2% slurry was significantly superior to all treatments and acquired typical blue cheese flavour as well as

good body characteristics earlier than control cheese. These results could be attributed to the high concentration of soluble nitrogenous compounds, free fatty acids and carbonyl compounds in blue cheese containing ripened slurry, since free volatile fatty acids and methyl ketones are major flavour of blue cheese (Jolly and Kosikowski, 1975; King and Clegg, 1979).

CONCLUSION

In this investigation an attempt was made to accelerate blue cheese ripening using ripened slurry and crude proteolytic and lipolytic enzymes secreted by *P. roqueforti*. These additives stimulated the reaction rate for the formation of important flavour compounds, such as free fatty acids, amino acids, and carbonyl compounds.

Addition of ripened blue cheese slurry or naturally produced proteolytic and lipolytic enzymes seems to be the most suitable method for accelerating cheese ripening. However, further studies concerning its application on an industrial scale should be investigated.

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