Journal of Agricultural Science and Food Technology
Vol. 3 (6), pp. 110-116, November, 2017
ISSN: 2465-7522
Full Length Research Paper
http://pearlresearchjournals.org/journals/jasft/index.html

Monitoring Proteolysis in Milk Caused by *Pseudomonas fluorescens* by the TNBS method

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Accepted 18 November,2017

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ABSTRACT

The objective of this study was to monitor proteolysis of milk during storage at 20 and 4° C by two strains of *Pseudomonas fluorescens* namely: NCIMB 702085 (416) and NCIMB 701274 (414) by the TNBS method. Since commercial *Pseudomonas fluorescens* enzymes are not available, live *Pseudomonas fluorescens* microorganisms were inoculated into milk followed by extraction and purification by dialysis. Bacterial enzymes obtained were then inoculated into UHT skim milk. Non-inoculated milk was used as a control. Results obtained showed that *Pseudomonas fluorescens* NCIMB 702085 was more proteolytic than *Pseudomonas fluorescens* NCIMB 701274. It was concluded that the TNBS method proved useful in monitoring proteolysis in milk by *Pseudomonas fluorescens*.

Keywords: Bacterial enzymes, dialysis, purification, UHT skim milk.

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INTRODUCTION

Refrigerated storage of raw milk is universally accepted for prolonging shelf life and preventing spoilage by mesophilic bacteria. Due to evolutions in the dairy market in which dairies have become more and more centralized, milk is now stored longer at refrigerated temperatures (Gaafar and Ali, 1995). *Pseudomonas spp*., the predominant psychrotrophic genus isolated from refrigerated raw milk and a major spoilage agent in the dairy industry (Sorhaug and Stepaniak, 1997). Psychrotrophic are the dominant microbiota of raw milk and are known to compromise heat-treated milk (e.g., UHT) due to the production of heat-stable enzymes during their growth in raw milk (Marchand et al., 2009). The extracellular proteases produced by these microorganisms, which degrade milk protein during storage under refrigeration are usually thermostable, keeping their activity even after pasteurization and UHT treatments (Champagne et al., 1994; Datta and Deeth, 2003; Fairbairn and Law, 1986; Kohlmann et al., 1991; Marchand et al., 2009; Oleiveira et al., 2015;Sorhaug and Stepaniak, 1997; Stepaniak and Fox, 1983). They can induce serious defects such as yellowish, green-to brownish, or blue coloration of cheese surfaces (Martin et al., 2011); poor development of ripening flora; and undesirable flavor and melting texture (Champagne et al., 1994; Ledenbach and Marshall, 2009), all of which are responsible for product depreciation and thus economic losses. Proteolysis in milk has been attributed to endogenous enzymes such as plasmin or exogenous enzymes such as bacterial proteases (Datta and Deeth 2001; 2003). The TNBS method was chosen since it was recommended for use in routine laboratory analysis on the basis of its accuracy, reliability, and simplicity (Chove et al., 2011). The present study aims to monitor

proteolysis by two strains of *P. fluorescens* and their enzymes by the TNBS method.

MATERIALS AND METHODS

Materials

Milk samples

Raw milk was obtained from the Centre of Dairy Research (CEDAR), University of Reading, UK. Commercial pasteurized and UHT skim milk was supplied by Dairy Crest, Shropshire, UK. Laboratory pasteurization (APV TRADEMARK, the APV Company LTD, UK) was carried out at the pilot plant in the Department of Food and Nutritional Science, University of Reading, UK. Unless otherwise stated, all experiments were carried out at the natural pH of the milk.

Bacterial strains

Two strains of *P. fluorescens* NCIMB 702085 and NCIMB 701274 were inoculated into skimmed pasteurised and UHT milk samples to study their effect on proteolysis. These strains were obtained from the departmental stock culture (Department of Food and Nutritional Sciences, University of Reading, UK), maintained at -80 °C. Prior to inoculation, they were grown overnight in nutrient broth followed by overnight agar slants at 30 °C. Streaking at 30 °C for 24 h was carried out to check the purity of samples followed by Gram staining. Both strains were grown on nutrient agar with CFS (cetrimide fucidin cephaloridine) at 30 °C for 24 h to confirm the fluorescence characteristics of *P. fluorescens*. Positive strains were incubated on plate count agar (PCA) at 30 °C for 24-48 h followed by microbial counting. An overnight culture was grown on nutrient broth and inoculated into skimmed UHT milk for the detection of proteolysis in milk.

Experiments

To study the effect of storage time on proteolysis, skimmed pasteurised and UHT milk samples were inoculated with *P. fluorescens* at 20 ºC for 7 days. To check the effect of storage temperature on proteolysis, experiment was conducted at 20 and 4 °C for 7 days. Gelation was monitored daily by checking the first appearance of clots. The pH was also monitored.

Clarification procedures

To obtain 6% TCA and pH 4.6 soluble extracts were carried out after incubation for all milk samples studied. Prior to clarification, all milk samples were heated and held at 100 ˚C for 10 min to denature the proteins. The 6% TCA soluble extracts were obtained by mixing equal volumes of TCA with milk and left to stand for 1 h before filtration through Whatman No. 41 filters. For isoelectric precipitation, the pH of milk was lowered to 4.6 by the addition of acetic acid and sodium acetate and left at room temperature for 1 h. The clear filtrate obtained was diluted with water to 100 mL (for pH 4.6 soluble extracts) but to 10 mL for 6% TCA soluble extracts. The clear extracts obtained were further filtered by 0.2 µm Millipore filter before being subjected to the TNBS method. The TNBS method was done as described by McKellar (1981). Triplicate samples of pH 4.6 soluble extracts (0.2 mL) were mixed with 2 mL 1 M-potassium borate buffer (pH 9.2) and 0.8 mL 5 mM-TNBS (Sigma-Aldrich, Gillingham, UK). After 30 min incubation in the dark at 25 °C, 0.8 mL freshly prepared 2 M-monobasic Na_2PO_4 [containing 18 mM-Na_2SO_3] and 5 mL distilled water were added. Absorbance was read at 420 nm by a spectrophotometer (Cecil CE 1021 1000 series Cambridge, England).

Extraction of *Pseudomonas fluorescens* enzymes

Bacterial strains were cultured into UHT skim milk and incubation at 37 ˚C for 3 days. Cells were removed by centrifugation (24,000 g for 10 min at 5 ˚C), resulting in a clear supernatant which contained the active enzyme. The crude enzyme extract was stored at -20 ˚C until further required. To purify the enzyme, the supernatant was dialyzed against distilled de-ionized water (with 0.05 % sodium azide) to prevent bacterial contamination. Ten mL of crude enzyme extract from each of the two strains were dialyzed in 14 kDa molecular weight cut-off tubular porous membrane (Medicell International Ltd., London, UK). The tubes were clipped securely and placed in 1500 mL of distilled water. Dialysis was carried out at 20 ˚C for 24 h, with one change of water. The semi-purified enzyme was cultured into UHT skim milk and incubated for 24 h. Samples without added enzymes were controlled. Samples were drawn after 2, 6 and 24 h and analysed by the TNBS method.

Statistical Analysis

Statistical analysis was carried out by using Statistical Package for Social Sciences (SPSS version 16, SPSS Inc., Chicago, USA). General Linear Model of analysis of variance (ANOVA) was used to determine statistical differences between means. LSD (Least Square Differences) and Duncan's multiple range tests were used to determine values that were statistically different ($p<0.05$). All analyses were carried out in triplicate, and results are expressed as mean $\pm$ standard deviation (SD).

RESULTS AND DISCUSSION

Effect of storage time on proteolysis caused by *Pseudomonas fluorescens*

Table 1. Absorbance by the TNBS method on pH 4.6 and 6%TCA extracts to study the effect of storage time on proteolysis of commercial pasteurised skim milk inoculated with 10^5-10^6 cfu/mL *P. fluorescens* NCIMB 702085 and NCIMB 701274 and incubated at 20°C for up to 7 days.

Days of of incubation	Treatments	Absorbance of pH 4.6 soluble extracts at 420 nm	Absorbance of 6% TCA soluble extracts at 420 nm
	Control (no microbes)	0.015 ± 0.002 (a) A	0.170 ± 0.003 (a) A
	P. fluoresens NCIMB 701274	0.017 ± 0.001 (a) C	0.216 ± 0.017 (b) C
day 0	*P. fluoresens* NCIMB 702085	0.041 ± 0.004 (b) F	0.230 ± 0.011 (c) F
	Control (no microbes)	0.020 ± 0.003 (c) B	0.176 ± 0.003 (d) B
	P. fluoresens NCIMB 701274	0.178 ± 0.005 (d) D	1.853 ± 0.027 (e) D
day 3	*P. fluoresens* NCIMB 702085	0.252 ± 0.007 (e) G	2.248 ± 0.009 (f) G
	Control (no microbes)	0.019 ± 0.003 (f) B	0.173± 0.005(g)AB
	P. fluoresens NCIMB 701274	0.242 ± 0.006 (g) E	2.335 ± 0.072 (h) E
day 7	*P. fluoresens* NCIMB 702085	0.287 ± 0.005 (h) H	2.504 ± 0.017 (i) H

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment: The experiment was repeated 3 times; N=9; TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20).

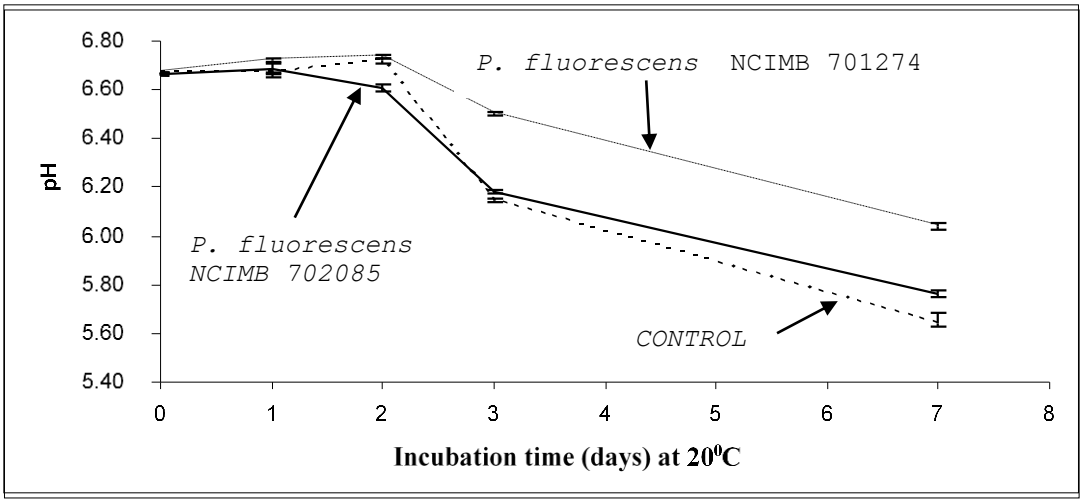

Figure 1.Changes in pH of *Pseudomonas fluorescens* NCIMB 702085 (416) and NCIMB 701274 (414) inoculated in pasteurised skim milk (10^5 -10^6 CFU/mL) during incubation at 20°C for 7 days; Error bars represent standard deviations; the experiment was repeated three times.

Table 1 showed that significant differences in proteolysis ($p < 0.05$) for all samples on days 3 and 7 in both soluble extracts studied. Results indicated that proteolysis in the inoculated samples was significantly different ($p<0.05$) for each bacterium on each day and in each soluble extracts. These samples were also statistically different from the control samples which had low proteolysis. The low but constant activity in the control sample was probably as a result of the activity of native enzymes (Datta and Deeth 2001; 2003). The high proteolysis of *Pseudomonas fluorescens* was also confirmed by its high absorbance on day 0 (Table 1), which was analyzed at about 30 - 40 min after inoculation. Gelation occurred on days 1 and 2 (data not shown), respectively for *Pseudomonas fluorescens* NCIMB 702085 (pH = 6.69) and NCIMB 701274 (pH = 6.73). Figure 1 also revealed that the pH was stable for 2 days (> 6.6) but after the third day, it fell steeply for all samples. Metabolites formed by *Pseudomonas fluorescens* caused the pH drop in the inoculated samples. The pH of the control sample dropped to 6.1, and this could be due microbial activity from the milk itself (Figure 1).

The pH 4.6 soluble extracts were well correlated to the 6% TCA soluble extracts as shown in Figure 2 (R^2 =0.989). This implies that both pH 4.6 and 6% TCA soluble extracts may be used to monitor proteolysis in milk during storage. Similar findings were reported by Gautcher et al., (2011) who found that an increase in pH 4.6-soluble nitrogen and trichloroacetic acid-soluble nitrogen content for UHT milk previously contaminated

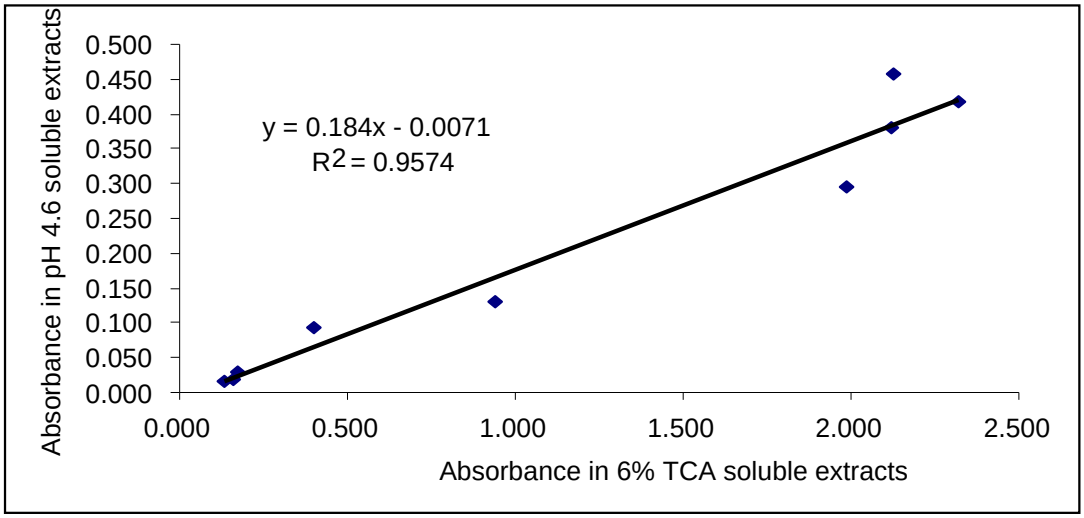

Figure 2. Correlation between pH 4.6 and 6% TCA soluble extracts from samples of UHT skim milk inoculated with 10^5 CFU/ mL *Pseudomonas fluorescens* NCIMB 701274 and NCIMB 702085 for 7 days at 20^0C as analysed by the TNBS method.

Table 2. Absorbance by the TNBS method on pH 4.6 and 6%TCA extracts to study the effect of storage time on proteolysis of commercial UHT skim milk inoculated with 10^5-10^6 cfu/mL *Pseudomonas fluorescens* NCIMB 701274 and NCIMB 702085 and incubated at 20 ˚C for up to 7 days.

Days of incubation	Treatment	Absorbance of pH 4.6 soluble extracts at 420nm	Absorbance of 6% TCA soluble extracts at 420nm
	Control (no microbes)	0.017±0.001 (a) A	0.132±0.004 (a) A
day 0	*P. fluoresens* NCIMB 701274	0.094±0.012 (b) D	0.397±0.002 (b) D
	P. fluoresens NCIMB 702085	0.130±0.035 (c) G	0.937±0.002 (c) G
	Control (no microbes)	0.019±0.002 (d) B	0.158±0.001 (d) B
day 3	*P. fluoresens* NCIMB 701274	0.296±0.002 (e) E	1.98 ±0.012 (e) E
	P. fluoresens NCIMB 702085	0.418±0.002 (f) H	2.32 ±0.183 (f) H
day 7	Control (no microbes)	0.029±0.002 (g) C	0.17 ±0.002 (g) C
	P. fluoresens NCIMB 701274	0.380±0.002 (h) F	2.12 ±0.043 (h) F
	P. fluoresens NCIMB 702085	0.458±0.004 (i) I	2.13 ±0.012 (h) I

Different letters (lower case) on the same column show significant differences ($p<0.05$) per day; Different uppercase letters on the same column show significant differences ($p<0.05$) per treatment: The experiment was repeated 3 times; N=9; TCA soluble extracts were diluted (x2), but pH 4.6 soluble extracts were diluted (x20).

were ten- and five-fold higher than those for control milk, respectively. The trichloroacetic acid-soluble nitrogen fraction of the milk contaminated before treatment contained 118, 22, 4 and 9 peptides from β-, αs_1-, αs_2- and κ-caseins in comparison to only 22, 19, 6 and 4 peptides for the control milk (Gautcher et al., 2011). As proteolysis by bacterial proteases produces smaller peptides and amino acids that are soluble in TCA (Lopez-Fandino et al., 1993), higher absorbance values reflect high activities from these bacteria. It has been documented that TCA precipitates large peptides as those formed by plasmin (Datta and Deeth, 2003). However, pH 4.6 soluble extracts contain peptides from both bacterial and native proteases (Datta and Deeth, 2003; Le et al., 2006) and hence their absorbance values also reflect bacterial proteolysis as no native proteases were added. Moreover, control samples had lower absorbance values, confirming that native proteases were probably too low to be detected (Table 1).

Table 2 showed that significant difference in proteolysis ($p < 0.05$) between all samples for all days of incubation except the inoculated samples from 6% TCA soluble extracts on day 7. Although significant differences in proteolysis were observed in these samples, *Pseudomonas fluorescens* NCIMB 702085 was the most proteolytic. These differences in proteolysis were probably due to the nature of the strains and particularly on the quantity of enzyme produced (Haryani et al.,

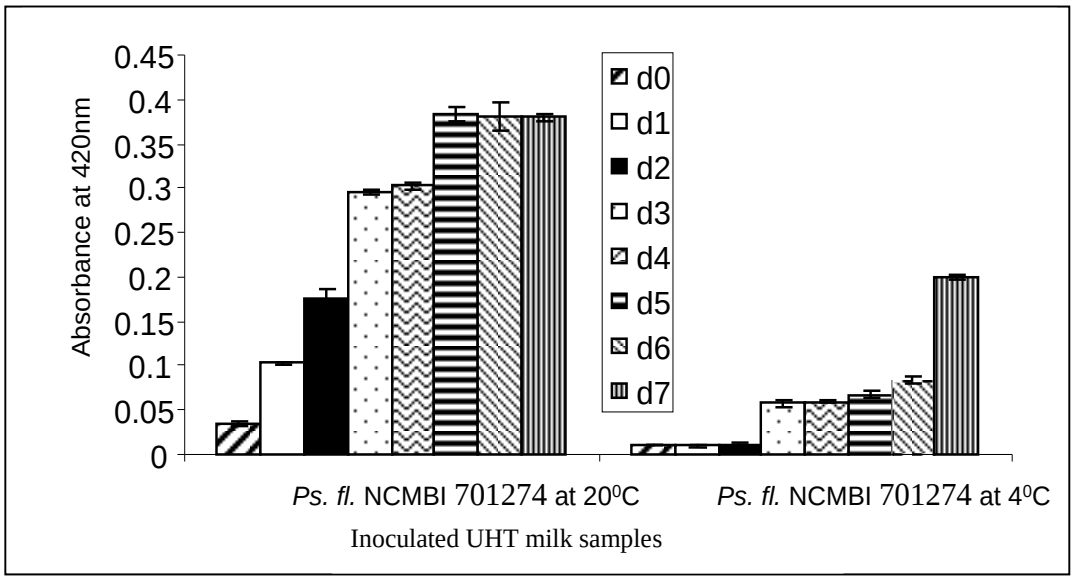

Figure 3. The effect of storage temperature on proteolysis by *Pseudomonas fluorescens* (Ps. fl.) NCMBI 701274 in pH 4.6 soluble extracts of UHT skim milk by the TNBS method. Error bars represent standard deviation; N=6; The experiment was repeated 2 times.

2003).

Many factors are involved in protease production and proteolysis. Although, there are contradictory reports regarding the quantity of enzyme produced and the activity of the microorganism, the strain of the bacteria was regarded as a crucial factor in determining proteolysis (Haryani et al., 2003).

Samples inoculated with *Pseudomonas fluorescens* NCIMB 702085 gelled after overnight incubation at 20 ˚C whereas *Pseudomonas fluorescens* NCIMB 701274 gelled after 3 days (data not shown). The control samples did not gel. This further suggests that the former strain (NCIMB 702085) was more active. A close relationship (R^2 = 0.957) between pH 4.6 and 6% TCA soluble extracts in UHT skim milk is shown on Figure 2, indicating that the TNBS method is an accurate method for studying proteolysis, using either extract.

Effect of storage temperature on proteolysis caused by *Pseudomonas fluorescens*

Pseudomonas fluorescens, a psychrotrophic, was expected to grow well under refrigeration and this was compared to room temperature (20 ˚C) where UHT milk is usually stored. Figures 3 and 4 demonstrated that the effect of storage temperature on proteolysis by *Pseudomonas fluorescens* NCIMB 702085 and *Pseudomonas fluorescens* NCIMB 701274 in pH 4.6 soluble extracts of milk. Both strains of *Pseudomonas fluorescens* have higher growth rate at 20 ˚C than at 4 ˚C (Figure 4). This implies that these microorganisms would cause more proteolysis at room temperature than under refrigeration. Other studies support these findings (Mitchell and Marshall, 1989; Nielsen, 2002; McKellar, 1981; Stepaniak and Fox, 1985). Morita (1975) confirmed that refrigerated storage has created selective conditions for the growth of psychrotrophic bacteria, which have optimal and maximal growth temperatures above 20°C but are still able to grow at low temperatures. Thus, although these microorganisms grow well under refrigeration temperatures, they can cause deteriorative changes at 20-30 ˚C which is their optimum temperature of activity (Nielsen, 2002). It is therefore important to observe hygienic handling of milk prior to storage to prevent microbial activities during storage.

Proteolysis caused by *Pseudomonas fluorescens* enzymes

P. fluorescens enzymes were dialyzed against water, so as to remove peptides and amino acids which would interfere with analysis. Addition of partially purified enzyme into milk is important because sodium azide, which controls the growth of microorganisms, can be used (Lichstein and Soule, 1944). Figures 1-4 shows UHT skim milk inoculated with dialyzed *P. fluorescens* NCIMB 701274 and NCIMB 702085 enzymes. It is evident that dialysis procedure further purified the samples since the breakdown products in dialysed samples were lower after 24 h than the non-dialysed samples. Figure 3 clearly showed that the effect of dialysis on purification of *Pseudomonas* spp., where

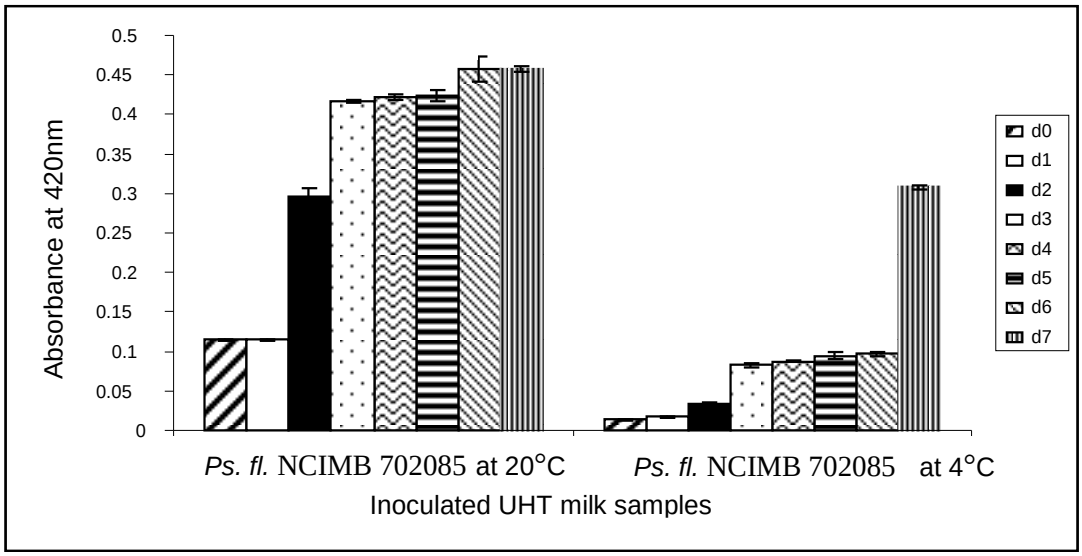

Figure 4. The effect of storage temperature on the proteolysis by *Pseudomonas fluorescens* (Ps. fl.) NCIMB 702085 in pH 4.6 soluble extracts of UHT skim milk by the TNBS method. Error bars represent standard deviations. N=6; The experiment was repeated 2 times.

Table 3. Absorbance of the pH 4.6 soluble extracts of UHT skim milk inoculated with dialysed *Pseudomonas fluorescens* NCIMB 701274 and NCIMB 702085 enzymes at 37 ºC for 24 h as analysed by the TNBS method.

	pH 4.6 soluble extracts			6% TCA soluble extracts		
Time (h)	Control	*Ps. fl*. NCIMB 701274	*Ps. fl*. NCIMB 702085	Control	*Ps. fl*. NCIMB 701274	*Ps. fl*. NCIMB 702085
0	0.024±0.001	0.026±0.002	0.038±0.002	0.107±0.008	0.184±0.003	0.201±0.011
2	0.022±0.003	0.106±0.007	0.187±0.005	0.140±0.014	0.504±0.005	0.836±0.018
6	0.035±0.003	0.121±0.004	0.191±0.004	0.131±0.011	0.580±0.019	0.942±0.026
24	0.038±0.005	0.231±0.015	0.350±0.007	0.141±0.021	1.212±0.040	1.539±0.078

* pH 4.6 soluble extracts were diluted (x 20) whereas 6%TCA soluble extracts were diluted (x 2).

absorbance readings are lowered by 60 and 72% *P. fluorescens* NCIMB 701274 and NCIMB 702085, respectively from pH 4.6 soluble extracts. The decrease of absorbance as a result of dialysis in the same figure is 73% and 80%, respectively for *P. fluorescens* NCIMB 701274 and NCIMB 702085 in 6% TCA soluble extracts, indicating the usefulness of this technique in partial purification of the enzymes. It is documented that enzyme purification by dialysis removes the peptides and amino acids that are associated with the enzyme preparation (Schokker and Van Boekel, 1997). It may be concluded that dialysis procedure is useful in removing the interfering substances.

Table 3 showed that for pH 4.6 soluble extracts, absorbance increased almost 5 times for *P. fluorescens* NCIMB 702085 and 4 times for *P. fluorescens* NCIMB 701274 between 0-2 h incubation. Proteolysis almost doubled between 6 and 24 h storage time for both strains. It may thus be concluded that significant changes in pH 4.6 soluble extracts absorbance occur in the first 2 h of incubation. The absorbance of the control sample was more or less constant throughout the incubation time and the pH was also constant (6.60 ± 0.020).

Gautcher et al. (2011) reported that the high level of proteolysis, observed for the UHT milk manufactured from raw milk contaminated with *P. fluorescens*, was confirmed by the increase in NCN (Non-Casein Nitrogen) and NPN (Non-Protein Nitrogen) contents in this milk compared to the control milk. The increase in NCN content was greater than the increases in NPN content suggesting that proteolysis favored the release of large casein fragments, present in the NCN fraction, but also small peptides and free amino acids present in the NPN fraction. This was in agreement with results reported by Celestino et al. (1997) and Rattray et al. (1997).

The 6% TCA soluble extracts show that *P. fluorescens* NCIMB 701274 increased by almost 3 times whereas *P. fluorescens* NCIMB 702085 increased by 4 times

between 0 and 2 h of incubation. Almost constant absorbance was observed between 2 and 6 h incubation for both strains of *P. fluorescens*. This observation appears to suggest that between 2 to 6 h, proteolysis is constant, however, after 6 h there is an increase in activity as observed by increased absorbance readings. Several studies supported that the heat-stable proteases retain their activity even after heat treatment. Griffiths et al. (1981) showed that proteases of different strains of *Pseudomonas* were able to retain about 20-40% of their activity after heat treatment to 140 °C for 5 s. Liao and McCallus (1998) indicated that over 20% of the enzyme activity persisted after a boiling for 10 min. Rajmohan et al. (2002) reported that a protease isolated from *P. fluorescens* was heat stable and retained activity even after treatment to 121 °C for 20 min. Marchand et al. (2008) showed that heating milk samples to 95 °C for 8.45 min resulted in a residual heat-resistant proteolytic activity of 73%.

CONCLUSIONS

This study provided an investigation of the effect of two strains of *P. fluorescens* and their enzymes on proteolysis of milk during storage. Comparison of the pH 4.6 and 6% TCA soluble extracts of UHT skim milk showed high correlation (R^2=0.9574) indicating that both extracts may be used to monitor proteolysis. Extensive degradation of caseins as a result of *P. fluorescens* growth observed in milk samples indicates that, even under refrigeration temperatures and storage, spoilage may still occur, affecting the safety of the consumers. The TNBS method was found to be a useful rapid method for monitoring proteolysis in milk. It is therefore recommended for routine assessment of proteolysis in the laboratories as it is considered to be simple and rapid compared to other traditional methods such as gel electrophoresis.

ACKNOWLEDGEMENT

The authors would like to thank the Association of Commonwealth Universities (ACU) for financial assistance for this study.

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