

Detection of Proteolysis in Milk by *Pseudomonas fluorescens* Using Urea PAGE Method

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Abstract

Proteolysis of milk during storage by two strains of *Pseudomonas* NCIMB 702085 (414) and NCIMB 701274 (416) was investigated using the Urea PAGE method. *Pseudomonas fluorescens* enzymes were also extracted and purified by dialysis before inoculation into UHT skim milk in an attempt to partially purify the enzyme. Results showed that dialysis removed some peptides and amino acids which would interfere with the assay procedure. The method also confirmed that *Pseudomonas fluorescens* NCIMB 701274 (416) was more proteolytic than *Pseudomonas* NCIMB 702085 (414). Thus, Urea PAGE is a useful method for monitoring proteolysis in milk by *Pseudomonas fluorescens*.

Keywords: Proteolysis, *Pseudomonas fluorescens*, Dialysis, Urea PAGE, UHT milk

1. Introduction

Proteolytic enzymes are of great importance to the dairy industry because they are responsible for imparting desirable or undesirable properties to dairy products through changes in flavour and texture (Datta & Deeth, 2003). Proteolytic enzymes that cause problems in milk and dairy products are of two major types, milk proteinases which naturally occur in all milk, and bacterial proteinases produced by contaminating bacteria (Nielsen, 2002). Proteolysis in refrigerated milk is usually caused by Gram negative psychrotrophs. Of these, *Pseudomonas spp.* are predominant.

Psychrotrophic microorganisms are predominant in raw milk microbiota during the storage, producing hydrolytic enzymes such as proteases and lipases which are responsible for many quality issues, for example; defects and deterioration, resulting in the limited shelf-life of dairy products (Chen et al., 2003). These hydrolytic enzymes detected in skim milk are of particular concern, indicating that they could be produced by *Pseudomonas sp.* during the cold storage of raw milk, contributing to the spoilage problem in milk and dairy products (Corre et al., 2011). Although psychrotrophs are usually destroyed by heat treatments, the extracellular endometalloproteases enzymes produced by these microorganisms are usually thermostable, keeping their activities even after pasteurization and even ultra-high temperature (UHT) treatments (Datta & Deeth, 2003; Kohlmann et al., 1991; Stepaniak, 2004). As a result, spoilage in milk may occur after pasteurisation and, more likely, after UHT heat treatment. Enzymatic spoilage without concomitant bacterial growth is of special concern in UHT milks (Gaucher et al., 2011). As these enzymes are not commercially available, one aim of this research was to extract them followed by partial purification before inoculation into UHT skim milk.

In this study, the Urea PAGE method was used to monitor proteolysis by two strains of *Pseudomonas fluorescens*, which were inoculated into milk.

2. Materials and Methods

2.1 UHT Milk

Commercial UHT skimmed milk was supplied by Dairy Crest, Shropshire, UK, and utilised within two weeks of buying.

2.2 Bacterial Strains

Two strains of *Pseudomonas fluorescens* (NCIMB 702085 and NCIMB 701274) were inoculated into skimmed UHT milk 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. 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 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 *Pseudomonas fluorescens*. Positive strains were incubated in 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. Samples were stored at 20°C for 7 days. Unless otherwise stated, all materials were from Fisher (Fisher

Scientific UK Ltd, Leicestershire, UK).

2.3 Urea PAGE

The protocol was based on Laemli (1970).

Samples and markers preparations

Samples: 100 μ L of samples were mixed with 400 μ L of reducing sample buffer. These were allowed to stand in boiling water for 2 min and allowed to cool to 25°C before loading into the gel.

Markers: Low molecular weight markers ranging between 6.5-66 kDa (Sigma-Aldrich Gillingham, UK) were prepared as follows: A vial containing low molecular weight markers was reconstituted with 100 μ L of deionised water. To ensure complete dissolution of the chemical, the vial was vortexed for a few seconds. The markers were dispensed in Eppendorf vials in aliquots and any unused portion was stored at -18°C until required. The proteins in the low molecular weight marker were as follows: albumin, bovine serum (66 kDa); ovalbumin from chicken egg (45 kDa); glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa); carbonic anhydrase from bovine erythrocytes (29 kDa); trypsinogen from bovine pancreas (24 kDa); trypsin inhibitor from soybean (20 kDa); α -lactalbumin from bovine milk (14.2 kDa) and aprotinin from bovine lung (6.5 kDa)

Reagents and solutions

Acrylamide-bisacrylamide stock solution (40% T): (Sigma-Aldrich Gillingham, UK): The solution was used directly from the bottle.

Separating gel buffer (0.5 M Tris, pH 8.8): Tris base (12.86 g) and 77.14 g Urea (77.14 g) was dissolved in 200 mL distilled water and pH adjusted to 8.8 with 5M HCl. The solution was stored at 4°C.

Stacking gel buffer (0.07 M Tris, pH 7.6): Tris base (1.66 g) was dissolved in 200 mL of distilled water. pH was adjusted to 7.6 with 5M HCl and stored at 4°C.

Electrode buffer: Tris base (1.5 g) and glycine (7.3 g) were dissolved in 1000mL of distilled water. No pH adjustment was made (the pH was 8.3). The solution was freshly prepared and cooled to 4 °C for at least 3 hours before use.

Reducing sample buffer: Tris base (0.75 g) and Urea (49 g) were dissolved in 100 mL distilled water and pH adjusted to 7.6 with 5M HCl. β -mercaptoethanol (0.7 mL) and 0.9 g of bromophenol blue were added. The solution was stored at 4°C.

Ammonium persulphate (10% APS): This solution was freshly prepared by dissolving 0.1 g of APS in 1 mL of distilled water.

Sodium Dodecyl Sulphate (10% SDS): SDS (10 g) was dissolved in 100 mL distilled water and stored at 25°C.

N,N,N',N'-Tetramethylethylenediamide- TEMED (Sigma-Aldrich Gillingham, UK): This was used directly from the bottle.

Staining solution: Coomassie blue G250 (1 g) was dissolved in 400 mL methanol, 100 mL acetic acid and the volume made up to 1000 mL with distilled water. It was stored at 25°C.

Destaining solution: To 400 mL of methanol, 100 mL of acetic acid was added and the volume made up to 1000 mL with distilled water

Preparation of the gel

Reagents	12% Separating Gel	4% Stacking Gel
Separating Gel buffer (mL)	7.0	-
Stacking Gel buffer (mL)	-	4.50
40% acrylamide solution (mL)	3.00	0.5
10% SDS (μL)	100.0	-
TEMED (μL)	10.0	5.0
10% APS (μL)	50.0	25.0
Total volume (mL)	10.160	5.030

Experiments:

Inoculation of Pseudomonas fluorescens into UHT milk to monitor proteolysis

The effect of storage time on proteolysis was studied at 37°C for 24 h. *Pseudomonas fluorescens* strains were inoculated into UHT skim milk followed by incubation at 37 °C for 24 h to monitor proteolysis. Control samples without added bacteria were also prepared. All experiments were carried out at the natural pH of the milk (6.6-6.7), unless stated otherwise.

Following proteolysis, breakdown products were analysed by Urea PAGE method. Prior to analysis, all milk samples were heated at 100°C for 10 min and held in a water bath (Grant Instruments Cambridge Ltd, Cambridge, UK) to denature the whey proteins. The Urea-PAGE protocol (Bio-Rad Labs, Richmond, California, USA) was used in which 100 μL of non-clarified samples were mixed with 400 μL reducing sample buffer. These were left to stand in warm water at 45°C for 5 min and allowed to cool in water to 25°C before they were loaded onto 12% gels. Staining was by Coomassie brilliant blue G 250 mixed with methanol and acetic acid, whereas de-staining was done by use of methanol and acetic acid.

This experiment was set up to monitor casein degradation following inoculation of *Pseudomonas fluorescens* 414 and 416 into UHT skimmed milk and incubated for 24 h but sampled at 2 and 24 h. Milk proteins may be identified by PAGE since the individual proteins can be separated according to size, or charge to mass ratio. This protocol was selected because the SDS-PAGE does not resolve the 4 caseins properly. The molecular weights of the 4 caseins are as follows: α_{s1}-caseins- 23 kDa; α_{s2}-caseins - 25 kDa; β- casein – 24 kDa and κ-caseins – 19 kDa (Fox, 2003). The Urea PAGE method is more appropriate because despite the close molecular weight range, it can resolve the casein accordingly. Separation by Urea-PAGE is based on charge to mass ratio.

Extraction and inoculation of Pseudomonas fluorescens enzymes into UHT milk to monitor proteolysis

A separate experiment was carried out where enzymes from both strains of *Pseudomonas fluorescens* NCIMB 702085 and NCIMB 701274 were extracted and cultured into UHT skimmed milk, followed by 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 prior to purification. To further purify the enzyme, the supernatant was dialysed against distilled de-ionised water

(with 0.05 % sodium azide). Dialysis was performed to remove peptides and amino acids from the enzyme, which would interfere with the assaying procedures. Ten mL of crude enzyme extract from each of the two strains of *Pseudomonas fluorescens* were dialysed 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 at 20°C for 24 h, with one change of water. The semi-purified enzyme was inoculated into UHT skimmed milk and incubated for 24 h. Samples without added enzyme acted as controls. Samples were drawn after 2, 6 and 24 h to be analysed by gel electrophoresis.

To determine the optimum enzyme concentration, various concentrations of the enzyme extracts [5%, 10% and 20% (v/v)] were inoculated into UHT skimmed milk prior to incubation at 37°C for 2 and 24 h followed by analysis by gel electrophoresis. The selected levels were based on a study by Lee (2006) who indicated that 5-25% enzyme extracts could be used for proteolysis. After preliminary studies (results not shown), a level of 10% enzyme extract was considered appropriate for further study, as readily detectable results were obtained.

Electrophoretograms were used for qualitative monitoring of the degree of proteolysis in milk by Urea PAGE method. No quantitative methods were employed in this study.

3. Results

Results obtained were as shown on Figures 1, 2 and 3. In Figure 1, milk samples which had been inoculated with the two strains of *P. fluorescens* and incubated for 24 h at 37°C were monitored. Initial results indicated that one strain was more proteolytic than the other. This is important information for milk samples during storage as some strains could result in spoilage within a very short time as compared to others. In Figure 2, UHT milk that had been inoculated with 5, 10 and 20% enzymes of one strain of *P. fluorescens* 414 and incubated at 37°C for 24 h was observed. This was done so as to find an optimum level of enzyme to be used for inoculation after dialysis. Figure 3 shows an electrophoretogram of 10% dialysed sample of *P. fluorescens* enzymes and its suitability in monitoring proteolysis by both strains of *P. fluorescens*.

4. Discussion

4.1 Proteolysis Caused by *Pseudomonas fluorescens*

Preliminary experiments indicated that the number of viable cells in the stock (inocula) from the departmental culture of *Pseudomonas fluorescens* was 10^7 - 10^8 cfu per mL. It was reported that a psychrotrophs population of 10^4 cfu/ mL is sufficient to induce proteolysis in milk (Renner, 1988). In the current experiments, about 10^5 - 10^6 cfu/ mL (1 % v/v) was inoculated into UHT skimmed milk to initiate proteolysis.

Storage time had a significant effect on proteolysis of the inoculated samples. The low but constant proteolysis in the control sample was probably due to native enzymes. Many factors are involved in protease production and proteolysis. It has been documented that, although there are contradictory reports regarding the quantity of enzyme produced and the activity of the microorganisms, the strain of the bacteria was regarded as crucial factor in determining proteolysis and not their number (Haryani et al., 2003; Hankin & Shields, 1983). Thus, milk with higher psychrotroph count did not necessarily have higher proteolysis than those with

lower counts. However, it was emphasised that minimum bacterial counts at which bacterial proteinases can be produced must be monitored (Haryani et al., 2003). Other researchers have suggested that the extent of proteolysis of milk proteins by enzymes depended on the number of target sites available for each enzyme and on the accessibility of these target peptide bonds by proteases. Moreover, control samples had lower absorbance values, confirming that levels of native proteases were probably too low to cause significant proteolysis.

Figure 1 shows that with increased incubation time greater hydrolysis of caseins was observed. The β -caseins were preferably hydrolysed at 2 h incubation, whereas α -s₁ and α -s₂ casein were also hydrolysed after 24 h. It was also obvious that *Pseudomonas fluorescens* 416 enzyme had faint bands of α -s₁, α -s₂ casein and β -caseins at all incubation times, indicating more degradation and higher enzyme activity than *Pseudomonas fluorescens* 414 enzymes.

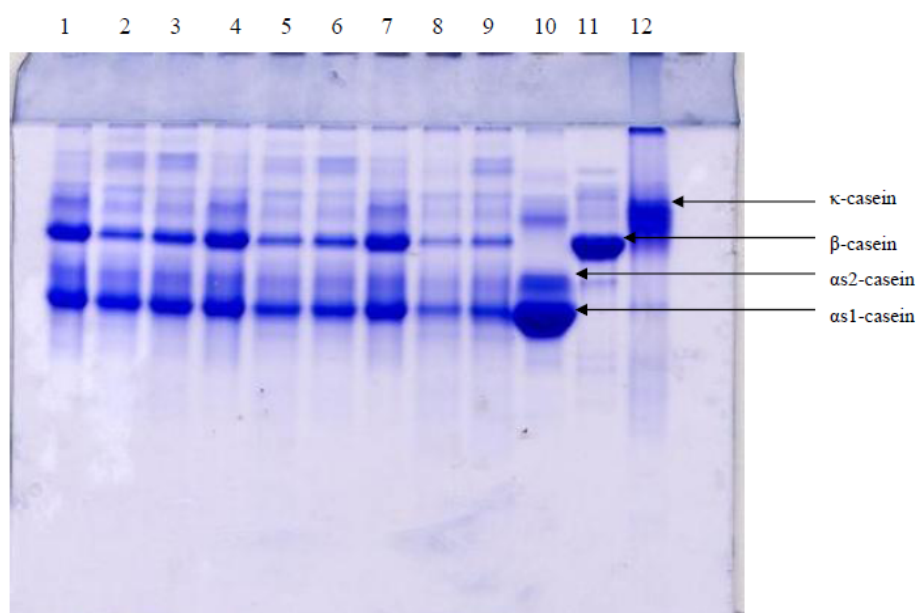


Figure 1. Urea PAGE electrophoretogram (T=12%, C= 4%) *Pseudomonas fluorescens* enzymes incubated in UHT skimmed milk at 37°C for 2 and 24 h

Legend: Lanes 1-3: *Pseudomonas fluorescens* 414 at 5%, 10% and 20% in UHT skimmed milk after 2 h incubation at 37°C. Lane 4: Empty lane Lane 5 – 7: *Pseudomonas fluorescens* 414 at 5%, 10% and 20% in UHT skim milk after 24 h incubation at 37°C. Lanes 8: empty lane Lanes 9-11: α - casein, β – casein, κ - casein and Lane 12: a mixture of α , β , κ caseins respectively.

The caseins in control samples were not degraded (lanes 1, 4 and 7). Comparison between inoculated samples and the controls confirmed preference for *Pseudomonas fluorescens* 416 (lanes 5 and 8) which degraded β -casein. There was also gradual disappearance of α s₁ and α s₂-caseins which was most evident after 24 h on lane 8. Although κ -casein has shown some degradation, its poor resolution in the gel makes it difficult to conclude on the extent of hydrolysis.

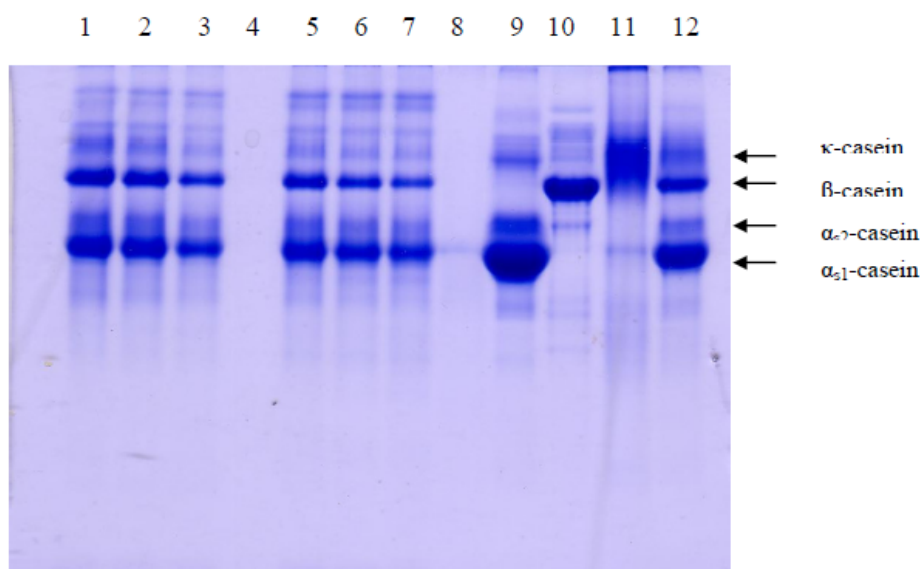


Figure 2. Urea PAGE electrophoretogram (T=12%, C= 4%) *Pseudomonas fluorescens* enzymes incubated in UHT milk at 37°C for 2 and 24 h

Legend: Lanes 1-3: *Pseudomonas fluorescens* 414 at 5%, 10% and 20% in UHT skim milk after 2 h incubation at 37°C. Lane 4: Empty lane Lane 5 – 7: *Pseudomonas fluorescens* 414 at 5%, 10% and 20% in UHT skim milk after 24 h incubation at 37°C. Lanes 8: empty lane Lanes 9-11: α- casein, β – casein, κ– casein and Lane 12: a mixture of α, β, κ caseins respectively.

Figure 2 shows the effect of incubating the various concentrations of *P. fluorescens* 414 at 5%, 10 % and 20% for 2 and 24 h at 37°C on proteolysis. As expected, the bands for *P. fluorescens* 414 were denser at 2 h incubation for all concentrations studied compared to 24 h of incubation indicating advanced levels of proteolysis. The α_{s1}-casein bands were denser than the β- and α_{s2}-casein bands, indicating preference of *P. fluorescens* to hydrolyse β-caseins over α_{s1} and α_{s2}-caseins especially after 24 h of incubation. Proteolysis was most evident at 20% enzyme concentration where both β- and α_{s2}-caseins were hydrolysed. The κ-casein band, though very faint, gradually decreased in intensity from lanes 1-6 confirming its hydrolysis by *P. fluorescens*.

In a study by Chove et al., (2011) analysis of proteolysis by plasmin enzyme on day 7, showed completely disappearance of both the α- and β-casein bands, indicating extensive proteolysis on these caseins. Microbial enzymes have been reported to show preference to hydrolyse κ-casein, which is usually found on the surface of the casein with the formation of para- κ-casein (Snoeren & Van Riel, 1979), followed by extensive hydrolysis which is non-specific (Law et al., 1977). Other authors have also documented that κ-casein is the ideal substrate for bacterial proteolysis (Fairbairn & Law, 1986; Datta & Deeth, 2003). However, in one study it was revealed that different strains of *Pseudomonas spp.* showed different hydrolysis rates on caseins whereby some degraded whole casein more than α , β or κ -casein while others degraded β –casein more than α and κ-casein (Mitchell & Marshall, 1989). A study of 6 proteases revealed that 3 were more active against α -casein than whole casein, γ, β or κ- casein;

and in another study, α -casein and whole casein were hydrolysed to the same extent (Patel et al., 1986). It was stipulated that whole casein could have a configuration that made it more susceptible to enzyme action (Mitchell and Marshall, 1989). The caseinolytic potential of these proteases is highly variable and strain dependent (Dufour et al., 2008). Some studies have been performed to understand the proteolytic activities on the milk proteins and especially on the casein molecules. Koka and Weimer (2000) indicated that a protease isolated from *P. fluorescens* RO98 preferentially hydrolysed κ -casein in artificial casein micelles. With the same objective, Costa et al., (2002) showed that an extract of *P. fluorescens* RV10 culture proteolysed both κ and β -casein. A study by Nicodème et al., (2005) characterized an extracellular protease from *Pseudomonas aureofaciens* LBSA1 and identified some peptidic bonds which had been cleaved by this enzyme on different purified casein molecules. However, all these studies were only performed on model systems with more or less purified enzyme and hence more studies need to be conducted using purified enzyme extracts.

It has also been stated that a great deal of variability observed in the ability of the proteases to hydrolyse κ -casein is due to conformational differences in proteins that can affect substrate specificity. As previously explained, the various strains of *Pseudomonas fluorescens* have different preferences for hydrolysing caseins. The γ -caseins are a result of the activity of native enzymes (presumably plasmin) on β -caseins. As expected, the bands are fainter after 24 h than after 2 h because of the longer time of incubation and hence enzyme activity.

4.2 Proteolysis Caused by Dialysed *Pseudomonas fluorescens* Enzymes

Figure 3 shows the effect of incubating dialysed concentrations of enzymes from NCIMB 701274 (414) and NCIMB 702085 (416) strains for 6 and 24 h at 37°C by Urea PAGE. Preference for β -casein degradation over α_{s-1} and α_{s-2} was evident for both strains especially after 24 h of incubation.

Incorporation of Urea into the samples permits the separation of most caseins and reveal the removal of phosphate groups from individual caseins (Bingham et al., 1976), and thus this method was recommended to monitor proteolysis of caseins during the ageing of cheeses. Hekken and Thompson (1992) observed that 40% dephosphorylation of whole casein slowed down the migration rate compared to the native β -casein. The authors explained that the removal of negatively charged phosphate groups slowed down the migration rate of the protein on the gels. The same researchers also found that κ -casein was poorly resolved in the gel, an observation which was also made in the current study.

The figure below shows the breakdown of proteins in milk by *Ps.fl.* enzymes

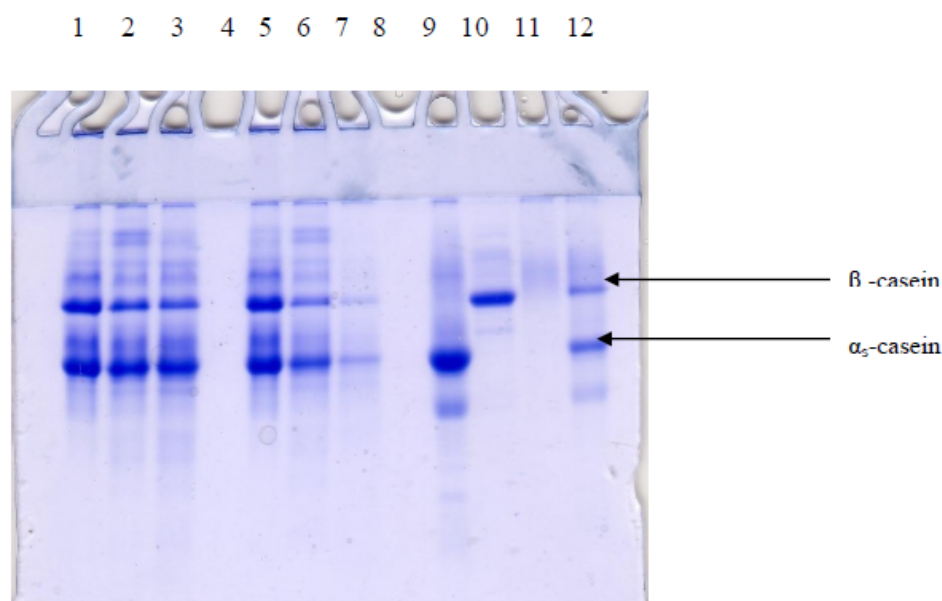


Figure 3. Urea PAGE electrophoretogram (T=12%, C=4%) of dialysed *Pseudomonas fluorescens* (10%) enzymes incubated in UHT milk at 37°C for 6 and 24 h

Legend: Lanes 1-3: Control (UHT milk) *Ps. fl.* enzyme 414 and *Ps. fl.* enzyme 416 at 6h
Lane 4: Empty lane **Lanes 5-7:** Control, *Ps. fl.* enzyme 414 and *Ps. fl.* enzyme 416 incubated for 24 h. **Lane 8:** Empty lane **Lanes 9 -11:** α -(s2&s1); β - and κ -casein respectively Lane 12: α -(s2&s1); β - and κ -casein.

UHT skimmed milk inoculated with dialysed *Pseudomonas fluorescens* 414 and 416 enzymes (10%), indicated that the dialysis procedure further purified the samples since the breakdown products in dialysed samples were lower after 24 h than the non-dialysed samples (Figures 1 and 3).

Inoculating the milk with live *Pseudomonas fluorescens* presented problems because other microorganisms could grow in milk as no sodium azide was added. Hence, *Pseudomonas fluorescens* enzymes were dialysed against water, so as to remove peptides and amino acids which could interfere with the analytical procedures. Addition of partially purified enzyme into milk is a useful tool because sodium azide, which controls growth of microorganisms, can be used. Hence, by adding Sodium azide, no additional bacterial proteases could be produced on storage and thus only proteases that survived treatments could act on caseins.

Enzyme purification by dialysis removes the peptides and amino acids that are associated with the enzyme preparation (Schokker & Van Boekel, 1997). Further purification of the *Ps. fl.* enzyme by ammonium sulphate precipitation was carried out by some researchers and this was found to increase the specific activity by 13 fold (Schokker & VanBoekel, 1997). This is recommended in future studies.

5. Conclusion

This study focused on the role of *P. fluorescens* and their enzymes on proteolysis in UHT

skimmed milk by Urea PAGE. Results showed that proteolysis increased with storage time. These properties are exploited in Urea-PAGE method.

Urea PAGE method is a useful qualitative method to observe breakdown profiles of caseins by microbial enzymes. *P. fluorescens* showed preference for degradation of β -casein over α_{s1} and α_{s2} caseins. It was also revealed that *P. fluorescens* 416 was more proteolytic than *P. fluorescens* 414, which was more evident after 24 hours of incubation.

Partial purification of *P. fluorescens* enzyme by dialysis was found to be an effective method in removing interfering substances and hence better method for monitoring proteolysis. Thus, it may be concluded that Urea PAGE method is a useful procedure for monitoring proteolysis in milk by both strains of *P. fluorescens*.

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