



PROCEDURES TO EVALUATE THE PROTEOLYTIC ACTIVITY IN RAW MILK AND THE EFFECT OF SUCH ACTIVITY ON THE ALCOHOL STABILITY OF PROTEINS IN RAW MILK

(PRJ-0090-2016)

University of the Free State

Year 2016 (January 2016 till December 2016)

Project goals

Objective 1 - Objective: Literature review. Goal: To do a literature review of rapid and sensitive screening techniques for microbial protease and plasmin activity in milk. Target dates: January 2016 – May 2016 but also adding new information as it becomes available

Achievements

Literature review was completed (See uploaded document).

Ms A Hattingh submitted her Masters thesis at the end of January 2017 and in the final revision the literature review was again updated.

No Non-achievements / underperformance has been reported

Objective 2 - Objective: Validation of methods for the determination of proteolytic activity in raw milk (Responsible student/person – Aninke Hattingh). Goal: To validate methods for effectively determining the proteolytic activity in raw milk and to distinguish between activity caused by addition of commercial bacterial proteases and milk plasmin. Target dates: March 2016-September 2016.

Achievements

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We treated raw milk with commercial bovine plasmin and protease from **Pseudomonas** and **Bacillus**.

The milk was precipitated using 12 % TCA and 2.5 M HCl. The Supernatant was analysed with RP-HPLC.

The plasmin and Bacillus peptide profiles on the HPLC of hydrolysed milk were undoubtedly not the same. (See report by Ms A Hattingh)

We also detected that the protease from **Pseudomonas** had no activity. The enzyme was replaced by Sigma.

We managed to get both **Pseudomonas** and **Bacillus** growing in UHT milk and it seems to

produce high level of protease activity according to the skim milk agar plates, the protease kit and the RP-HPLC chromatography hydrolysis peptide profiles (It is important to note that with the exception of the proteases kit all the other tests present results on qualitative bases and not on quantitative bases). During the next phase on RP-HPCL work we are going to attempt to group all the prominent peaks for each enzyme type together and use the HPLC software to integrate the series of peaks as such. The RP-HPLC data (peaks) for commercial Bacillus and the self-cultivated Bacillus protease compared favourably with one another.

A biochemist (Dr D. Shabott) working on software development helped us with the programming of software that enabled us to point out profile differences between plasmin and microbial protease RP-HPLC profiles. We already imported a number of HPLC runs into the software can with the limited number of profiles available already give a 100% prediction if the proteolytic action is due to microbial protease or due to the indigenous plasmin action.

During my earlier study years, I worked with agar plates with the relevant substrates present in it in order to identify microorganisms that can produce the relevant enzyme to break down the substrate produced by microbial strains during a screening process. When I learned about milk agar plates we started use it to evaluate it to detect protease and plasmin activity. It really works just fine and but still need some more fine tuning (See Ms A Hattinghs progress report).

The (68% alcohol) Alizarol and the protease activity tests were evaluated on various milk samples by the Ms A. Hatting and our lab is now set up to run these test on a routine basis (Also see Ms Hattinghs progress report).

No Non-achievements / underperformance has been reported

Objective 3 - Objective: Application of selected tests for determining the proteolytic activity in raw milk during incubation of milk samples at 7 oC until the end of the shelf-life. Goal: To use selected tests for the determination of proteolytic activity to determine the the proteolytic activity in raw milk during a period of shelf-life incubation at 7 oC until flocculation as determined by the Alizarol test. Target dates: April 2016- end November 2016.

Achievements

The first part of this objective (the collection and incubation) was performed by prof. C. Hugo and her research group.

They collection of raw milk samples from Dairy Corporation located in Kwaggafontein directly West of Bloemfontein next to the main Kimberley road. After collected the raw milk samples were incubated at 7o C until all the milk samples tested positive with the Alizarol (72%) test. Due to that fact that this milk samples at this stage had a high microbial and enzyme load, the samples were immediately frozen and stored at -25oC until further study. Due to the cost implication (RP-HPLC, R18000) it was necessary to cut down on the numbers of samples to be analysed as far as possible. Therefore we limited the study to only the final milk samples which will be protease positive beyond any doubt.

The second part of this objective was to take these Alizarol positive raw milk samples and to analyse them using the various developed chemical tests in order to demonstrate the sensitivity and also to point out the presence of bacterial proteases and milk plasmin within the samples.

By using the RP-HPLC it was relatively easy to detect that

Non-achievements / underperformance

I would have preferred to trace the protease production in raw milk samples as from day one up and until week six.

Reasons for non-achievements / underperformance

The enormous increase in the price of commercial enzymes form 2015 to 2016 totally depleted our budget. Some chemicals like the MilkoScan standards that was budget for in the 2016 budget, could not be order due to the lack of funds. The commercial enzyme price increased by more than 120% from 2015 to 2016 and but because the enzyme forms an integral part of this study we need to order it all cost.

Finally the money left in the entity was divided by the unit price for the

microbial protease was active in the milk due to the position of prominent peaks within the peptide profiles (Due to the complex ability nature of the various peaks on RF-HPLC chromatograms it was not possible to quantify them). It was even possible to identify that *Pseudomonas* was the dominant bacterium during cultivation (part one) based on its characteristic chromatographic peptide profile liberated from the milk. The microbiological work done by prof Hugo and her students on the same milk samples also confirm the presents of the bacterium *Pseudomonas* after 6 weeks of cultivation.

(Take note that the above is extraordinary conditions and will not occur normally within the fresh milk chain)

The Alizarol test was not performed on the collected samples due to fact that it was already performed during the first part of this work by prof Hugo student.

The protease assay kit from Merck gave really good and repeatable results that also confirmed the presents of proteases in the milk samples. Apart from the fact that the technique is little bit expensive (R7000 for 100 samples, thus R70 per sample) it is very simple to perform if you have access to an UV spectrophotometer in your analytic facility. A drawback is that even-though it gives quantitative values, the activity is nonspecific (Bacterial protease/Plasmin).

The milk agar plate's results are outstanding even though the techniques still needs to be optimized during 2017. The results (halos) obtained were clearly visible within a short time span of less than an hour. It was also possible from previous work done to distinguish between the halo periphery formed by plasmin action and microbial protease action. The Plasmin gives a clear zone, were as the microbial zone of hydrolysis was visibly milky. The best part of this technique is its simplicity (do not need a trained person to perform the test) and its affordability.

Finally Conclusions about the various tests:

Nonspecific: (Cannot differentiate between microbial and plasmin activity)

The Alizarol test works well but is very subjective to the operator's interpretation and totally none specific (it gave a flocculation positive test at time zero before any damage could have taken place to the casein).

The protease assay kit is capable of defining the protease level, but is nonspecific.

Specific: (Has the capability to differentiate between microbial and plasmin activity)

RF-HPLC has the capability to differentiate between plasmin and microbial damage (We intent to get more peptide profiles from more flocculation causing bacteria during 2017). This test is very sensitive and can even identify the bacteria responsible for protein damage. I hope that soon we can modify this technique to provide quantitative results (In theory it must be possible but in laboratory it might not work). Even though this test is not cheap, it is the only test that on its own can address the flocculation problem. The source that is causing the flocculation being it on farm or factory can easily be identified and corrective action can be taken.

RP-HPLC and the experiment was planned accordingly. This work was also the final work done at the end of the year.

The unrest on campus the last few months of 2016 really also complicated the situation and we sometimes had to be escorted by police to the laboratories to perform duties during these unfortunate times and occasionally we could not enter the campus at all.

Ms. Aninke Hattingh submitted her Master's thesis on this work at the end of January 2017.

The milk agar test is very simple and during the optimization stage (2017) we intent to make the test very simple to enable the farmers to evaluate his/her raw milk at farm level. Due to the differences of the perimeter of the clearing zone (Halos) it is possible to identify the presents of plasmin or bacterial protease in a positive test.

Income and expenditure statement

Income and expenditure statement	SCX-4623_20160531_08204201.pdf Oktober Copy of Finansiële verslag Melk SA (002).xlsx Finansiële state vir 2016.zip Finansiële verslag Melk SA vir 2016.xlsx Milk SA signed report 2016.pdf Asset register and location.xlsx
Unnecessary spending during period	No

Popular Report

[Populere Vorderings verslag final Eiende 2016.docx](#)
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Additional documentation

[Populere Vorderings verslag.docx](#)
[Milk flocculation Aninke.docx](#)
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Statement

Levy funds were applied only for the purposes stated in the contract	Yes
Levy funds were applied in an appropriate and accountable manner	Yes
Sufficient management and internal control systems were in place to adequately control the project and accurately account for the project expenditure	Yes
The information provided in the report is correct	Yes