Interest of the use of feather in the generation of news strains isolated from rotten bovine hides

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1. Introduction

CTC has conducted an innovative program to evaluate the interest of emerging technologies for calf skin unhairing. Based on a partnership with a laboratory specialized in biotechnologies and in enzyme and microbial engineering, this study describes the screening and isolation of a new strain, producer of proteases to be used to replace traditional chemicals.

2. Material and method

2.1.Luria Bertani plate:
This methodology is based on G Bertani works (1). LB medium is composed of Tryptone, 10 g/l; yeast extract, 5 g/L; NaCl, 10 g/l ; pH 7,2. Agar is added is the solution at 17g/l. It is sterilized in an autoclave during 20 minutes at 121°C before running tests.

2.2. Sampling of strains:

When hairs start to fall from the hide, the top surface of the hide is scraped. The sample is picked up and is grown on LB plate at 37°C during 48 hours.
The different strains obtained are transferred in 5 ml of LB in liquid form and agitated during 24 hours at 37 °C at 160 rpm in order to maximize their growth.

2.3. Skimmed milk:

This method is adapted from Ernst H. Reimerdes, Henning Klostermeyer (2). Each strain previously mentioned is picked up in flasks containing 1,5g of Agar and 50mL of nutrient broth is autoclaved at 121°C during 20 minutes. Before using the medium, skimmed milk is warmed at 50 °C. 50 ml of skimmed milk is then mixed with nutrient broth under sterile conditions. A drop of culture medium is spread on the plate forming a central wale. Proteolytic activity is detected when a translucent halo is formed on the plate.
The strains that have a proteolytic action are then transferred into 5 ml of liquid LB during 24 hours and then diluted in a glycerol solution and frozen in cryotub at – 20°C.

2.4. Control of keratinolytic activity:

Feather culture based on a study of Fakhfakh-Zouari, N., Haddar, A., Hmidet, N., Frikha, F., Nasri, M. (3)

- A medium based on feather is prepared with 10 g/l of feather, 0.1 g/l of $MgSO_4$, 0.5 g/l NaCl, 0,7g/L de KH_2PO_4 et 1,4g/L de K_2HPO_4, completed to 1 liter with water. It is then autoclaved at 121° C during 20 min, in order to be a sterile conditions.
- 5 ml of the pre-culture (which has proteolytic action) are transferred into a 500 ml flask, in which 100 ml of feather medium is placed.
- After an incubation of 24 hours at 30°C and 160 rpm, the culture medium is centrifuged during 10 min. The supernatant obtained is then sterilized through a 0.2 µm filter.

2.5. Keratin azure:

Based on a method developed by Radhika Tatineni *et al.* (4), the supernatant extracted from the previous manipulation is then tested with keratin azure.
4 mg of keratin azure substrate are incubated during 30 min at 55°C with 500 µL of supernatant and 500 µL of buffer Tris-HCl at pH7 50 mM.
The mixture is then centrifuged at 10000 g during 5 minutes. The absorbance is then measured at 595 nm. One enzymatic activity unit is defined by the absorbance variation of 0.01.

Unhairing activity:
The supernatant is placed on a piece of skin to check if there is an unhairing activity.

2.6. Identification of the strain:

Sequencing of RNA 16S:
The RNA sample amplified through PCR is introduced in a sequencer (analysis realized by Eurofins MWGoperon). The sequence obtained is then entered in a database (by a blast on NCBI database) .

2.7. Identification of the protease:

Analysis by FPLC (Fast Protein Liquid Chromatography)
The support G4000 for gel filtration chromatography was used to screen components that have big molecular weights, like proteases.
Each protein was eluted and showed a "peak" which has been collected for further use.
By running several times this operation, it is possible to concentrate the protein eluted in order to launch further analyses.
The effluent concentrated containing protein is then test on a piece of skin.

If the test works, then it is analyzed through ion exchange chromatography, and then to mass spectrometry analysis to obtain characterization informations of the protein.

3. Results and discussion

3.1.Sampling of strains:

After 4 days of hide putrefaction, we obtained a substrate in which bacteria are shown (figure 1).

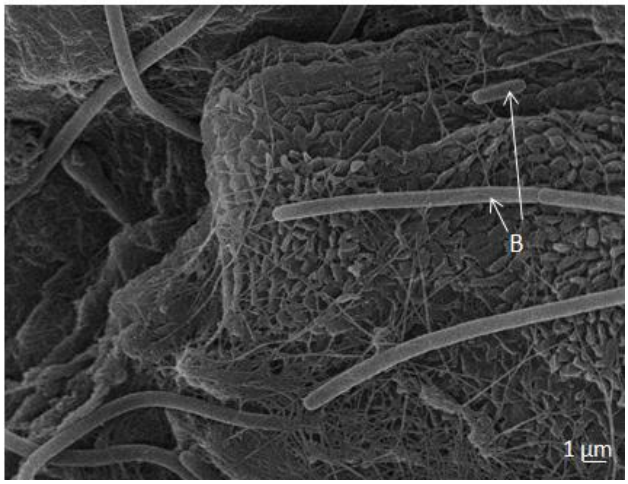

Figure1: Scanning Electronic Microscopy of the hide surface (SEM)/B: bacteria

3.2. Control of proteolytic activity:

From LB plate broth, we obtained 6 strains.
Once they were selected through skimmed milk, only one strain presented a proteolytic activity, observed by the formation of a translucent halo formed on the plate.

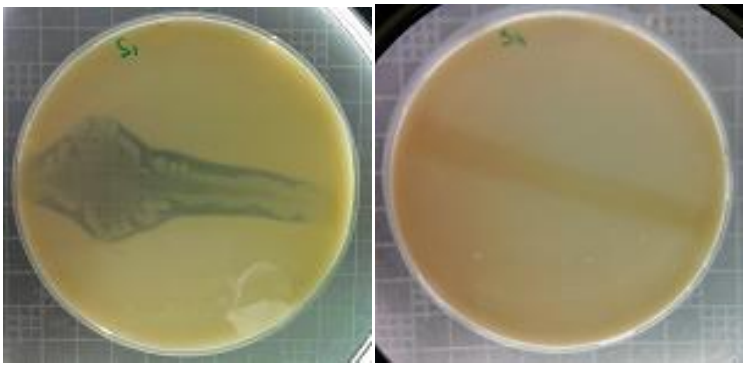

Figure 2: Skimmed milk plate: S1(left): translucide halo which shows proteolitic activity ; S4(right): negative witness

3.3. Control of keratinolytic activity:

Once proteolytic strain was selected, we checked if it had a keratinolytic activity. The supernatant obtained after the culture in a feather mixture was centrifuged and filtered on a 0.2 µm filter. It showed activity through keratin azure test method.

In the analysis conducted, we have measured an enzymatic activity of 8.4 U/ml (or 16.8 U/ g skin).

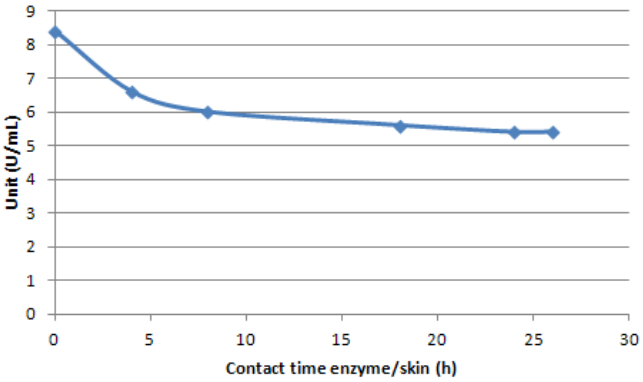

Figure 3: Effect of the time on the enzymatic activity

This supernatant was also tested on a piece of raw skin and we have obtained an unhairing activity without grain abrasion.

3.4. Test on the skin:

Four hours after the contact with the skin, there is a beginning of action.

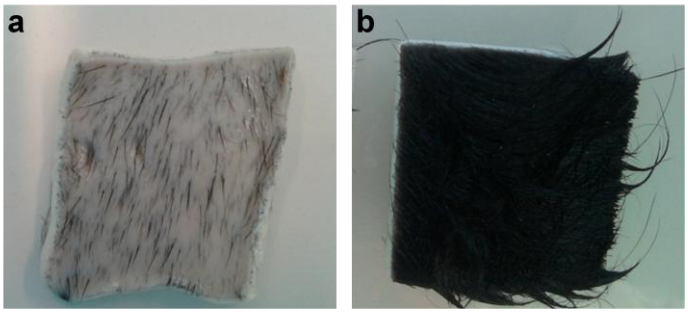

Figure 4: Test conducted with the supernatant
(a). A standard assay was conducted with the feather medium
(b) without enzymatic activity.

3.5. Identification of the strain:

After checking that the sequence obtained after PCR amplification contains RNA, (figure 5), we have confirmed that it is a RNA, because the band has 1500 pairs of basis. 16 s RNA is then sequenced and we launched the analysis on NCBI database.

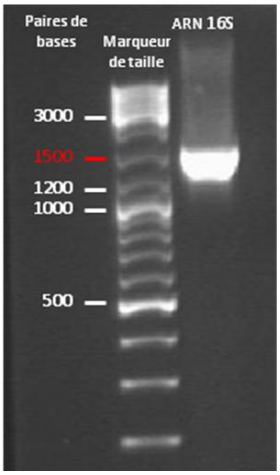

Figure 5: Agarose gel which shows ARN 16 S migration of the strain analyzed.
Size marker : « O'GeneRuler » from Fermentas.

ARN 16 S analysis able to detect a *Bacillus Cereus***.**

3.6. Purification of the strain and identification of the protease responsible of unhairing activity:

The supernatant obtained from the feather culture was analyzed by FPLC.
Gel filtration was firstly used to fractionate the supernatant. Four fractions were recovered and analyzed.

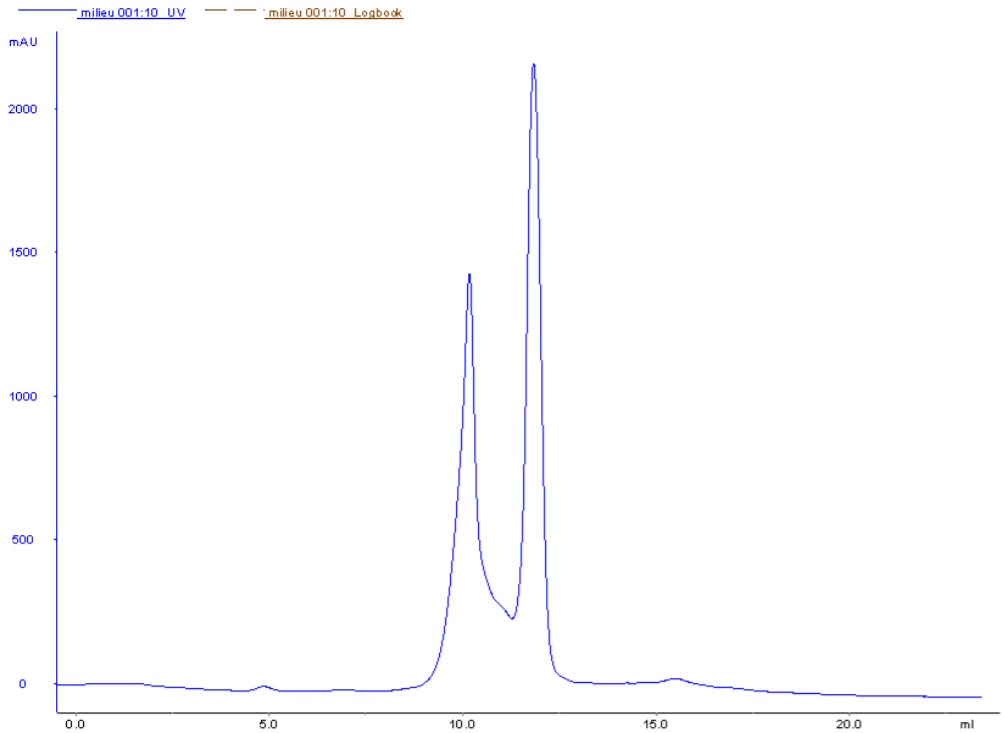

Figure 6: Chromatogram obtained with FPLC system

The tests conducted showed that the sample obtained from this 4 components did not showed unhairing activity. We hypothesized the low concentration of the sample or and the problem of support not suitable for separation of the mixture proteins in FPLC system.

Currently, we focused on other methods such as electrophoresis to analyze more precisely the pure enzyme responsible of this unhairing activity.

4. Conclusion

Microorganisms were removed from raw hides. Samples were directly plated on Luria-Bertani agar plates to isolate the different strains.

After isolation, plates containing skimmed milk and nutrient broth were used for microorganism selection. Then microorganisms were cultured in a medium containing feather to promote the production of keratinolytic enzymes.

One strain shows proteolytic activity on skimmed milk plates and was grown in feather medium. After culture, the supernatant was tested on a small piece of skins and show unhairing activity.

We have isolated a strain of *Bacillus cereus* from raw hides that presents a good unhairing capacity on calve skins. Enzyme purification is currently studied.

References

(1) Studies of lysogenesis
The mode of phage liberation by lysonigenic *Escherichia coli*.G. Bertina- Department of Bacteriology, University of Illinois, Urbana, Illinois. Received for publication May 14, 1951

(2) Determination of proteolytic activities on casein substrates Original Research Article
Methods in Enzymology, Volume 45, 1976, Pages 26-28
Ernst H. Reimerdes, Henning Klostermeyer

(3) Application of statistical experimental design for optimization of keratinases production by *Bacillus pumilus* A1 grown on chicken feather and some biochemical properties Fakhfakh-Zouari, N., Haddar, A., Hmidet, N., Frikha, F., Nasri, M. (2010) *Process biochemistry*. 45, p617-626

(4) Purification and characterization of an alkaline keratinase from Streptomyces, Radhika Tatineni et al. (2008) sp. Bioressource technology. 99, p1596-1602 ,