International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Production, Encoding Genes Variability and N-Terminal Sequences of Fibrinolytic Enzymes Produced by Bacillus Strains Isolated from Fermented Cassava Leaves "*Ntoba Mbodi*" at Brazzaville, Republic of Congo

Faly Armel Soloka Mabika[1-3], Etienne Nguimbi[1-2-3], Raoul Ampa[1], Aime Christian Kayath[1-2-3], Alain Vouidibio[1], Gabriel Ahombo[1-2]

[1]Laboratoire de Biologie Cellulaire et Moléculaire BCM, Faculté des Sciences et Techniques, Université Marien NGOUABI
[2]Institut de Recherche en Sciences Exactes et Naturelles (IRSEN)
[3]Unite de Biologie Moléculaire et de Bioinformatique, Faculté des Sciences et Techniques, Université Marien NGOUABI
Corresponding Author :
Etienne Nguimbi, MD, PhD, etienne.ng1612@gmail.com,
Faly Armel Soloka Mabika, MD, armelmabika@gmail.com
Raoul Ampa, PhD, ampa_ampa@yahoo.fr
Aime Christian kayath, PhD, chriskayath@yahoo.fr
Alain Vouidibio, PhD, vouidibio333@gmail.com
Gabriel Ahombo, PhD, , ahombogaby@gmail.com

Abstract: ***Nine (9) Bacillus strains isolated from Ntoba Mbodi, already characterized by their 16S rDNA and listed as: Bacillus safensis NM1, Bacillus safensis NM5, Bacillus pumilusNM28 Bacillus pumilus NM29, Bacillus subtilis NM39, Bacillus subtilis NM41, Bacillus megaterium NM56, Bacillus licheniformis NM74 and Bacillus amyloliquefaciens NM75, have been explored for Production, encoding genes variability and molecular characterization of their fibrinolytic enzymes. All of them produce proteolytic enzymes using casein as a substrate. Using fibrin, two of them as Bacillus safensis NM1, Bacillus safensis NM5 could not produce fibrinolytic enzymes. For the same conditions fibrinolytic enzymes production was different as was the growth. To explore encoding fibrinolytic enzymes genes variability, DNA was extracted and using three selected sets of primers in the literature, PCR amplification of corresponding genes was done with the optimization of annealing temperature (58°C, 60°C and 62 °C). Only three strains including. Bacillus subtilis NM39, Bacillus subtilis NM41and Bacillus licheniformis NM74 displayed specific bands in the 1% Agarose Gel electrophoresis for one to another set for 58°C. Sequencing of specific bands in two strains Bacillus subtilis NM41 and Bacillus licheniformis NM74 was performed, followed by a bioinformatic analysis. The gene encoding fibrinolitic enzyme in B. subtilis NM41 exibits 99% of similarity with subtilisin gene of B.subtilis G1, accession number EF061457.1 in GenBank. While that encoding fibrinolitic enzyme in B. licheniformis NM74 exibits 98% of similarity with Alkaline protease (apr) gene of B.licheniformis MP1, accession number HM147766 in GenBank. All the two fibrinolytic enzymes have some modifications in the pre, the pro and the mature peptide. The N-terminal sequence has shown in the two mature peptides, for the twelve first amino acids two kinds: AQTVPYGIPLIK for NFE-1 (Ntoba Mbodi fibrinolytic enzyme-1) of Bacillus licheniformis NM 74 and AQSVPYGISQIK for NFE-2 (Ntoba Mbodi fibrinolytic enzyme-2) of Bacillus subtilis NM41. The sequences of fibrinolytic enzymes in Bacillus strains of Ntoba Mbodi comprise the two kinds of organizations in N terminal sequences which are already described.***

Keywords: Production, gene variability, fibrinolytic enzymes, Bacillus, Ntoba Mbodi, N-Terminal sequence

1. Introduction

Production of proteolytic enzymes (proteases) is a normal physiological function of many organisms including bacteria. Besides their use in normal physiology, proteases are used in various industries including pharmaceuticals, detergents, food, and waste processing. Of all industrial enzymes used worldwide, proteases alone constitute nearly 60% [**1, 2].** Fibrinolytic enzymes are proteolytic enzymes which hydrolyze fibrin and casein. The fibrinolytic enzymes were successively discovered from different microorganisms, the most important among which is the genus Bacillus from traditional fermented foods. The physiochemical properties of these enzymes have been characterized, and their effectiveness inthrombolysis in vivo has been further identified. Therefore, microbial fibrinolytic enzymes, especially those from food grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure thrombosis and other related diseases [**3].**

Bacillus natto producing NK was the first screened from a traditional Japanese soybean-fermented food named natto [**4].**Over the years, other bacilli have been discovered to produce fibrinolytic enzymes.they are B. amyloliquefaciens DC-4from Chinese soybean-fermented food [**5, 2]**, Bacillus sp.CK from Korean fermented-soybean sauce [**6].**

Ntoba Mbodi is a popular fermented food in the Republic of Congo where it constitutes a significant source of protein in

the diet of the consumers. It is obtained by fermenting cassava leaves **[7].**It is essential toferment cassava leaves, as the process eliminates or decreases significantly the presence of toxic components such as cyanogenic compounds found in the raw material [**8, 9].** Moreover, the fermentation process allows the release of nutritious elements such as essential amino and fatty acids as well as vitamins. During the process, a riseof pH to a value up to 10 is observed; thus the product is classified as an alkaline fermented food. In such types of product, the main microorganisms responsible for the fermentation are Bacillus species, such as B. subtilis, B. licheniformis, B. amyloliquefaciens, B. pumulis, B. sphaericus, B. cereus, B. xylanilyticus, with B. Subtilis commonly reported as the predominant species [**10, 11, 12].**

Molecular characterization of Bacillus strains using 16S rDNA have been hold **[7].**Among the isolated strains, some of Bacillus strains as been tested as fibrinolytic enzymes producers. In a preliminary study, monitoring of growth and enzyme production have been discussed. It was established that for the sames conditions enzyme production vary from one strain to anotoher **[13].** Genetic diversity of fibrinolytic enzymes in Bacillus strains of Ntoba Mbodi have not yet achieved.

In the present work we explore production and genetic diversity of fibrinolytic enzymes in nine (9) Bacillus strains isolated from Ntoba Mbodi, These strains are already characterized by their 16S rDNA and listed as: *Bacillus safensis NM1, Bacillus safensisNM5, Bacillus pumilus NM28 Bacillus pumilus NM29, Bacillus subtilisNM39, Bacillus subtilis NM41, Bacillus megaterium NM56, Bacillus licheniformis NM74 and Bacillus amyloliquefaciensNM75.*

2. Materials and Methods

Bacterial strains and culture conditions

The Bacillus strains used in his study available in our laboratory were isolated from Ntoba Mbodi and already characterized by their 16S rDNA [**7].** They are listed as: *Bacillus safensis NM1, Bacillus safensisNM5, Bacillus pumilus NM28, Bacillus pumilus NM29, Bacillus subtilisNM39, Bacillus subtilis NM41, Bacillus megaterium NM56, Bacillus licheniformis NM74 and Bacillus amyloliquefaciensNM75.* Strains were grown in Luria-Bertani (LB) broth for 48 h at 37 °C.

Proteolytic enzyme production

Bacillus cells were cultivated in LB at 37°C with shaking. At the appropriate growth stage, the culture was centrifuged with a centrifuge VWR-MICRO STAR17Rat 4000t/minute for 5 minutes. The supernatant was assayed for caseinolytic activity by using casein plate **[14, 15].** It was modified as following:1g of agarose was dissolved in 250 ml erlenmeyer containing 100 ml de PBS at 0, 01N, after boiling, we waited until à 55-60°C, 5 ml 0, 2% of casein was added. The mixture were poured in petri dishes. Wholes were made on the plate. 50µl of sample were added in each whole, and the plate was incubated at 37°C for 12 hours. Caseinolytic activity was measured by lytic area through the diameter of the clear zone [**16, 17].**The growth was measured by optical density using a Zuzi spectrophometer Model 4211/50.

Fibrinolytic enzyme production

Fibrinolytic production was evaluated through enzyme assay**.** Fibrinolytic activity was determined using the fibrin plate method with modification **[16, 18].** Supernatant from centrifugation of appropriate growth stage of Bacillus cells was used. Succinctly; 25ml of 0.5% fibrin solution was mixed with 25ml of 1% agarose gel in petri dish and put for 30 minutes at room temperature. Wholes were made on the plate. 20µl of sample were added in each whole, and the plate was incubated at 37°C for 16 hours. Fibrinolytic activity was measured by lytic area through the diameter of the clear zone. Diameter of clear zone were measured and used to evaluate enzyme production.

In all enzymes activities, *E.coli K12,* was used as a negative control in extracellular enzyme activity, there is no clear zone when supernatant of cultivated E.coli K12 is put whatever in casein or fibrin plate

Encoding fibrinolytic enzymes genes

Primers design

Gene encoding fibrinolytic enzymes in bacteria display diversity as illustrate by different sets of primers used for different strains. According to the literature, we selected some primers which were already used to amplify encoding fibrinolytic enzymes genes in Bacillus strains. Three sets of primers used are listed in **Table I.**

Table I: Primers used in the present study.

N°	Primer sequence	References
Set1	P1: 5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC -3'F	**[19]**
	P2: 5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3' R	
Set2	**P3** 5′-TCACAGCTTTTCTCGGTC-3' F	**[20]**
	P4 5′-TGATCCGATTACGAATGC -3' R	
Set3	P5: 5'-ATGATGAGGAAAAAGAGTTTTTGGC-3'	**[21]**
	P6: 5'-CATCCGACCATAATGGAACGGATTC-3'.	

PCR amplification of encoding fibrinolytic enzymes genes and sequencing

Genomic DNA of all strains was isolated and used as a template [**13, 22].**. To amplify DNA fragments encoding fibrinolytic enzyme genes in all Bacillus strains, optimization was made using three sets of primers according to the literature (**Table I**),. PCR was performed using a GeneAmp 2400 PCR system (Perkin Elmer, Wathman, MA, USA). The mixture was prepared in a total volume of 50µl containing 30ng of genomic DNA, 150nmol/L each primer, 0.25mmol/L dNTP, 1.5mmol/LMgCl2, PCR buffer and 2.5U Taq polymerase. PCR conditions were for each set of

primers as following: first denaturation at 94°C during 5 min, 30 cycles comprising each : denaturation at 94°C for 25 sec., annealing at (58°C, 60 °C and 62°C), for 30 sec., and extension at 72°C for 40sec., final extension at 72°C for 5 min. The PCR product was purified using QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany).The sequencing was achieved by electrophoresis on a 3730xl DNA Analyser-Titania (Applied Biosystems) using the same primers in the **Table I**. Only two bands from two Bacillus trains (*B. subtilis. NM41 and B. licheniformis NM74*) were sequenced..

Softwares and Sequences analysis

To analyze enzyme production we used excel. Fibrinolytic enzyme genes were compared with homologs sequences in GenBank/EMBL/DDBJ Sequence database using the Basic Local Alignment Tool (BLAST) program (National Center for Biotechnology). Translation of fibrinolytic enzymes genes were hold with SMS-ORF finder ((National Center for Biotechnology).Sequences alignment were performed with Clustalw and MUSCLE.

To make sequences analysis, we firstly used Blastn to search for similarities with the homologs of already sequenced fibrinolytic enzyme genes. **The Table II** gives the information related to the score, percentage of identity (similarity), the type of the gene and the equivalent strain in NCBI-GenBank with the accession numbers.

As we know that it is about Coding sequences, we used Sequence Manipulation Suite (SMS) ORF Finder. ORF Finder searches for open reading frames (ORFs) in the DNA sequence you enter. The program returns the range of each ORF, along with its protein translation. Use ORF Finder to search newly sequenced DNA for potential protein encoding segments. ORF Finder supports the entire IUPAC alphabet and several genetic codes. **[23].**For the two genes sequences which encodes the fibrinolytic enzyme in two Bacillus strains (*NM41* and NM74), we searched for Open reading frames in frame 1, 2 and 3 on the direct and reverse strand, using the genetic bacterial code.

3. Results

1) Proteolytic enzyme production

In **Figure 1 (a** and **b)**, is showing the proteolytic enzyme production correlated with optical density (growth), when the substrate is the casein.

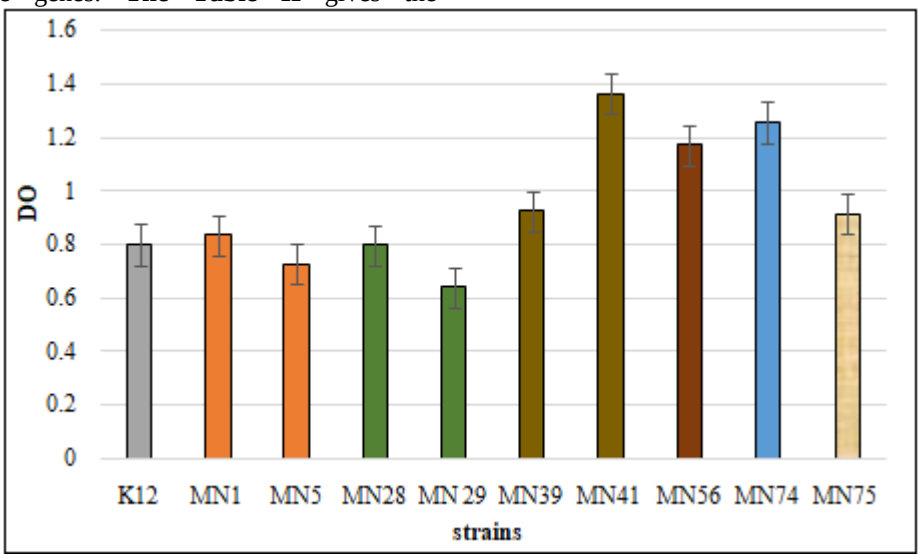


Figure 1a: Growth profiles of Bacillus strains isolated from Ntoba Mbodi. K12 is E.coli strain

Figure 1a shows clearly the variation of optical density. At 37°C for 48 hours, for all Bacillus strains, growth is different from one strain to another, growth is more important in *Bacillus subtilis*, *Bacillus licheniformis* sand *Bacillus megaterium* and less important in *Bacillus pumilus*. By anyway growth is observed in all strains.

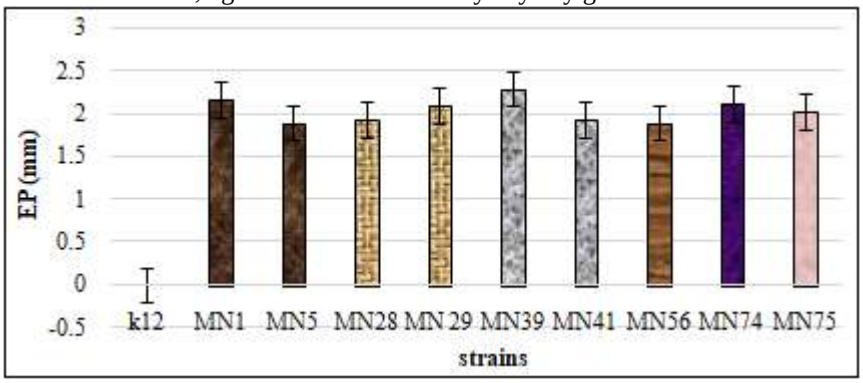


Figure 1b: Profiles of Proteolytic enzyme production (EP) in Bacillus strains isolated from Ntoba Mbodi. K12 is E.coli used as a negative control

Proteolytic enzyme production is observed in all used Bacillus strains. The enzyme production is more important in *Bacillus subtilis*, *in Bacillus licheniformis* and *Bacillus pumilus*. For two Bacillus subtilis strains, enzyme production is different. Enzyme production specific for each strains.

2) Fibrinolytic enzyme production

Figure 1c illustrates the fibrinolytic enzymes profiles.

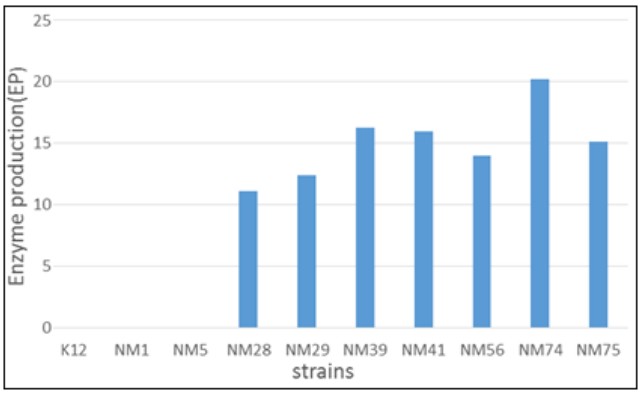
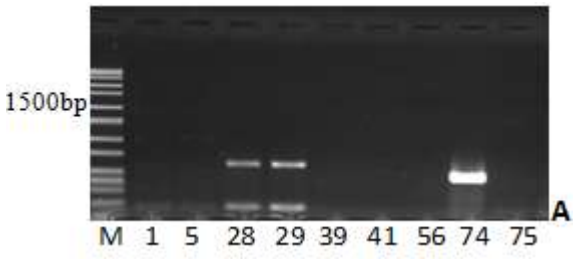


Figure 1 (c): Profiles of Fibrinolytic Enzyme Production (EP) in Bacillus strains isolated from Ntoba Mbodi. K12 is E.coli used as a negative control.

Bacillus licheniformis (*NM74*) has the most important enzyme production. The two *Bacillus subtilis* strains (NM39 and NM41) have significant enzyme production. The two *Bacillus safensis strains* (*NM.28* and*NM.29*) have not produce fibrinolytic enzyme at these conditions.
OD is used to express growth.

3) Agarose Gel Electrophoresis of PCR amplification of encoding fibrinolytic enzymes genes in Bacillus strains isolated from Ntoba Mbodi.

In **Figure 3** is shown the Agarose Gel Electrophoresis of PCR products.



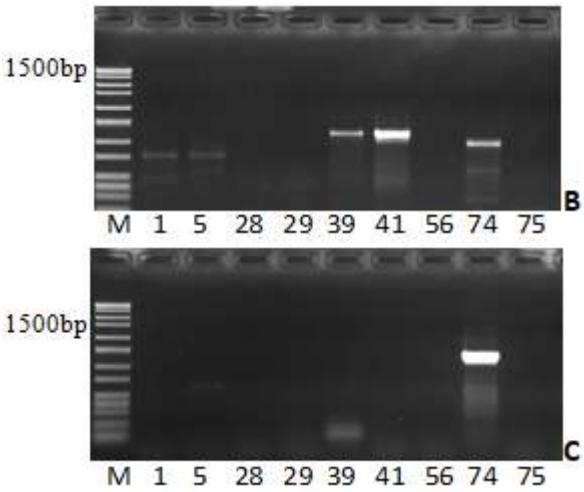


Figure 2: 1% Agarose Gel Electrophoresis of PCR amplified encoding fibrinolytic enzymes genes in Bacillus strains. **A:** with the primers P1 and P2**. B:** with the primers P3 and P4, **C:** with the primers P5 and P6. In **A, B** and **C**the order of samples were as following: Marker (M), NM1, NM5, NM28, NM29, NM39, NM41, NM56, NM74, NM75. NM1=*B. safensis*, NM5=*B. safensis*, M 28=*B. pumilus*, NM29=*B.pumilus*, NM39=*B.subtilis*, NM41=*B.subtilis*, NM56=*B.megaterium*, NM74=*B.licheniformis* NM75=*B.amyloliquefaciens*.In **A**, **B**and **C** the annealing temperature is 58°C.

In figure 3, the agarose gel electrophoresis of PCR amplified encoding fibrinolytic enzymes genes in Bacillus strains shows clearly in A, B and C a specific band of *Bacillus licheniformisNM74*. For the three different set of primers used, fibrinolytic enzyme gene in this strains is amplified. In figure3 (A), the two strains of *Bacillus pumilus* display two bands, these results were consistently obtained after optimization of annealing temperature, this may be non-specific bands. In figure 3 (B), the two strains of *Bacillus subtilis (NM39, NM41)* display a specific band with the same size in the two strains. In figure 3 (C), only the *Bacillus licheniformisNM74* display a specific band. Whatever the set of primers used, the size of all the PCR products corresponding to the amplified fibrinolytic enzyme gene in Bacillus strains isolated in Ntoba Mbodi is between 1500bp and 1200bp.

4) Sequences Analysis

After sequencing of encoding fibrinolytic enzyme genes, we used Blastn-NCBI to search for similarities among the homologs. **Table II** gives the information related to sequences similarities.

Table II: (Blastn -NCBI) results for the two sequences of gene encoding fibrinolytic enzymes in respectively *B.subtilis* NM41 et *B.licheniformis 74*

Strains	Max score	Querycover	E value	Percentage of.similarity	Accession/Souche NCBI	Type of gene
B.s NM41	2036	98%	0.0	99%	EF061457.1 / B.s. G1	Subtilisin gene compl. cds
B.l.NM74	857	96%	0.0	98%	HM147766/B.l MP1	Alkaline protease (apr) gene compl.cds

The table II shows clearly the sequence similarity of the two Bacillus strains, compared with the GenBank equivalent strains. The accession number and the type of gene are given. Subtilisn gene and alkaline protease (apr.) gene display a high similarity with the two sequences of respectively NM41 and NM74.

Table III: differences of nucleotides in encoding fibrinolytic enzyme gene among some Bacillus strains

Strains	Nucleotides sequences						
B.l.NM74	GAT	GGC	ATT	CAG	CGA	TTC	CGC
B.s.NM41	GCT	TGT	TGT	TTG	CGT	TAC	GTT
B.s.NATT	GTA	TGA	AAA	TAG	TTA	TTT	CGA
Ba sp. C4 SS-2013	CGA	GTC	TCT	ACG	GAA	ATA	GCG
B.s 50a	ATG	AGA	AGC	AAA	AAA	TTG	TGG

The differences in the beginning of encoding fibrinolytic enzyme genes in are clearly shown clearly Table III

It is important for coding sequences to compare the protein sequences. In Using Suite Manipulation Sequence-Open Reading Frame finder [**23].** For the translation of the sequence and Blastp-NCBI, the two sequences of NM41 and NM74 was very similar, with some differences in the pre, pro and the nature peptide. According to Blastp-NCBI results, the two fibrinolytic enzymes sequences display 99% of similarity with the nattokinase, which is the reference. In **Figure 4** similarity of fibrinolytic enzymes sequences is very evident, It is shown that the two sequences of the bacillus strains displayed a high similarity with others, but they have also some differences in the pre, pro and mature peptide. In the mature peptide, the first difference is at the third position in which all others contain a serine residue, as it is for the B.l.NM74, but the B.s.NM41 contains a threonine residue. The second substitution in the mature peptide, is in position nine there is a proline residue in the *B.l NM74* sequence, while it is a serine residue in the B. s.NM41.

These results are suggesting two kinds of organization in the N-terminal sequences of Bacillus strains in Ntoba Mbodi. *B.l.NM74* has a fibrinolytic enzyme which sequence is (AQTVPYGIPLIKA.), while *B.s.NM41* fibrinolytic enzyme sequence, is (AQSVPYGISQIKA).

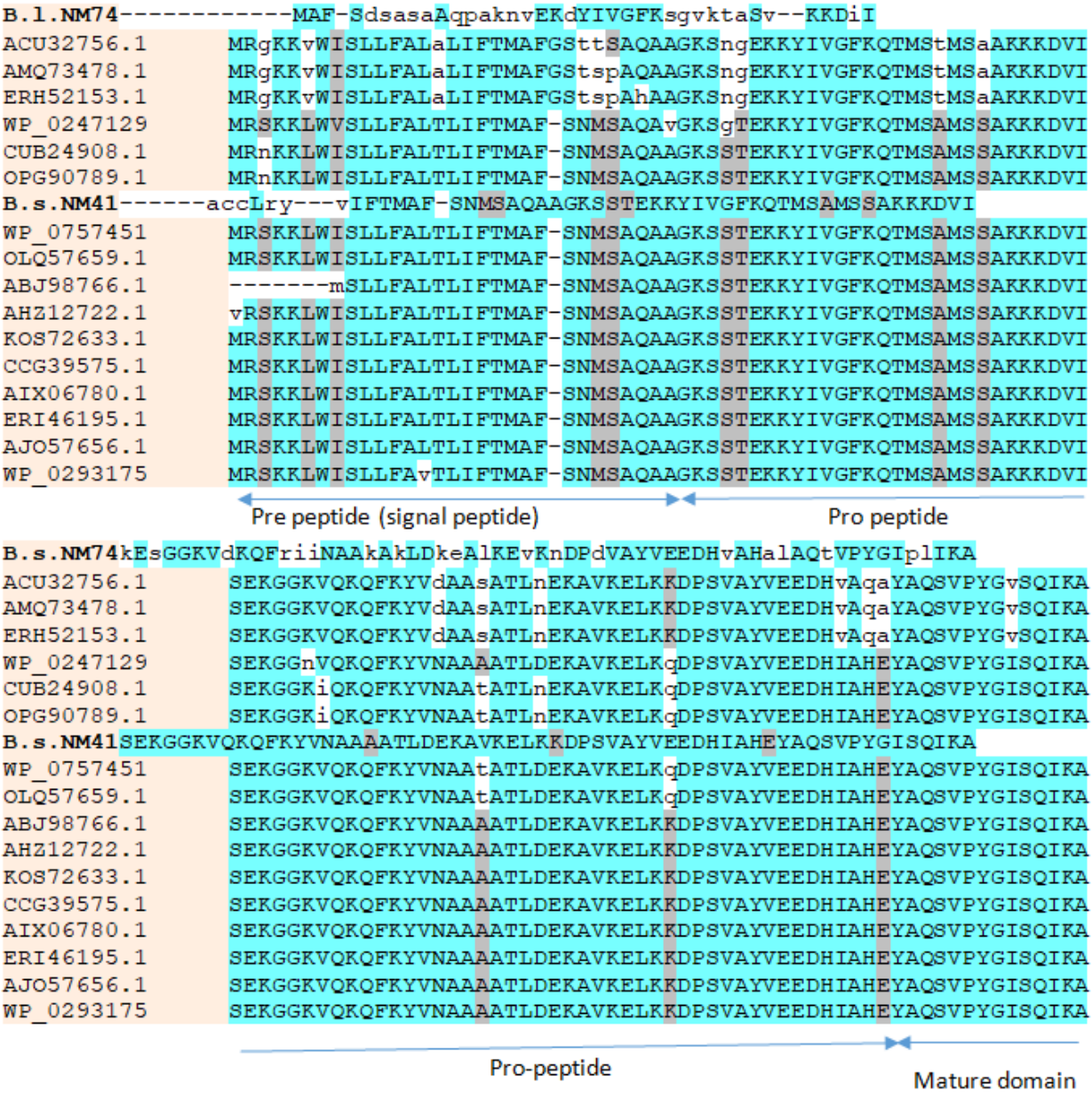


Figure 3: Part of Sequences alignment of fibrinolytic enzymes

Similar residues are colored as the most conserved one (according to BLOSUM62). Average BLOSUM62 score: Max: 3.0 Mid: 1.5, Low: 0.5. Two bacillus strains isolated from Ntoba Mbodi are: *B.l. NM74* with **NFE-1** and *B.s. NM41* with **NFE-2**.Others are homologs, from the Protein Bank.

Figure 3, is confirming that; in the pre, the pro and the mature peptide B.l.NM74 and B.s.NM41 have many substitutions

4. Discussion

Usually fibrinolytic enzymes are enzymes which hydrolyze casein and fibrin, that is the reason on which many are using casein to test fibrinolytic activity as it is for proteololytic activity.

This study explore fibrinolytic enzymes production, encoding gene variability and N terminal sequences in bacillus strains isolated from the Congolese alkaline fermented cassava leaves ¨Ntoba Mbodi˝. During the past decades, microorganisms demonstrating fibrinolytic activity have been isolated from fermented foods [**24, 25].** At present, various health foods containing nattokinase have been developed commercially [**26].** The fibrinolytic enzymes produced by these food-grade microorganisms are of special interest in developing functional foods beneficial to public health [**26].**. In this respect, this study has shown producing-fibrinolytic-enzyme-Bacillus strains isolated from the Congolese alkaline fermented cassava leaves¨ Ntoba Mbodi˝.

About production of fibrinolytic enzyme, we used fixed condition at 37 for 48hours to enhance the growth process; growth is different for each strain as it is for the enzyme production. Growth is observed for all strains. Except the E. coli K2, which is considered as a negative control on the fibrinolytic, all strains produce proteolytic activity. In most of bacillus strains fibrin and casein are hydrolyzed. But in two strains of *Bacillus pumilus*, we have an important caseinolytic activity but no fibrinolytic activity. In figure 2, *B.licheniformis NM74* has the most important fibrinolytic enzyme production, this is also important for the two Bacillus subtilis strains (NM39 and NM41). Diameters of clear zone have been the parameter used to evaluate fibrinolytic enzyme production. This parameter has been used by [**27].** they have the *Bacillus cereus* with a strong fibrinolytic activity which the clear zone is 11mm. Growth and enzyme production depend on the type of bacteria. The two different phenomenons are under control of culture conditions. At the same culture conditions, all strains have different growth profiles, and different enzyme production profiles. This study has given the results which are in concordance with that rulers. Many authors have claimed these rulers, like [**3, 28, 29].**

Encoding fibrinolytic enzymes genes in the two Bacillus strains (*NM74 and NM41*) were observed in the beginning of genes and compared with three other genes from GeneBank. In the Table III, we have the results of comparison. These results are showing how primers can be different, when the encoding the pre- peptide is used for primer design. To screen for encoding fibrinolytic enzyme gene in *Bacillus amyloliquefaciens* [**19, 20].**have used different primers for different results. [**19]** have also used another set of primers. In contrast all of the three sets of primers can amplify the encoding fibrinolytic enzyme gene in *B. licheniformis NM74.*

When the strain is identified by its 16S rRNA gene and classify in a bacillus group, it is better to use the high similarity of fibrinolytic enzyme sequences for designing primers.

In Figure 4, it is obvious that the sequence similarity is very high among fibrinolytic enzymes. I the pre peptide, *B.l.NM74* has a DSASA sequence, while most sequences have NMSAQ, these substitutions may play an important role in the synthesis of the enzyme. In the pro peptide, NM74 has a sequence SQVKTA, while most of sequences have QTMSAM.
In the mature peptide, three groups are very constant, the first one comprise the *B.l.NM74*, which has a threonine residue on the third (AQTVPYGIPLIK), the second group possesses a serine residue on the third position and a valine residue on the position eight., the third group has a serine residue on the third position, and an isoleucine residue on the position eight. The Strain *B. sutililis NM41* belongs to this third group. Consider the mature peptide, the same results have been already discussed by (**[30, 31, 32].**They have found the same N-terminal sequence of the mature peptide (AQSVPYGISQIK) for three different fibrinolytic enzymes: Subtilisin DFE, 31-kDa enzyme, Subtilisin QK-2 respectively.

These results are consistent with our **NFE-1** (Ntoba Mbodi-Fibrinolytic –enzyme -1), produced by *B.licheniformis NM74*. While the mature peptide N terminal sequence (AQTVPYGIPLIKAD) of **NFE-2** (Ntoba Mbodi – Fibrinollytic –enzyme-2), produced by *B.subtilis NM41*, is similar to CK which was found by [**19].**One of the remarkable substitution on the N-terminal sequences of *B.l. NM74* and *B.s NM41*, is on the third position, a serine residue for one but a threonine residue for another. Both are neutral polar, and having an alcohol function, the substitution could be a conservative one.

5. Conclusion

This study is a first one to clarify about the encoding fibrinolytic enzyme genes in Bacillus strains isolated from Ntoba Mbodi (Congolese Alkaline fermented cassava leaves). Fibrinolytic Enzyme Production have been clearly explored, encoding fibrinolytic enzyme gene variability is discussed and provide insight information in bacillus strains for Ntoba Mbodi. At last two new N-terminal sequences of Fibrinolytic enzymes (**NFE-1** and **NFE-2**), have two kinds of pattern organization which are similar to those found to other bacillus isolated in some fermented food. High similarity and few conservative substitutions of the N-terminal sequence of Bacillus will open a new way to design degenerated primers, for screening encoding fibrinolytic enzyme genes in Bacillus strains,

References

[1] Nascimento WCA, and Martins MLL (2004). Production and properties of an extracellular protease from thermophilic Bacillus sp. Braz. J. Microbiol., 35:91-96

[2] Do ThiBich Thuy and Salil Kumar Bose (2011). Characterization of Multiple Extracellular Proteases Produced by a *Bacillus subtilis* Strain and Identification of the Strain.*International Journal of Biology* Vol. 3, No. 1.

[3] Yong Peng.XiaojuanYang.Yizheng Zhang (2005).Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivoApplMicrobiolBiotechnol 69: 126–132

[4] Sumi H., Hamada H., Tsushima H., Mihara H., Muraki H. (1987). A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese natto; a typical and popular soybean food in the Japanese diet. Experientia. 43, 1110–1111.

[5] Peng Y., Zhang Y.Z. (2002.). Isolation and characterization of fibrinolytic enzyme-producing strainDC-4 from Chinese douchi and primary analysis of the enzyme property, High Technol. Lett., 12 (2) 30–33

[6] Kim, W., Choi, K., Kim, Y., Park, H., Choi, J., Lee, Y**.,** Lee H., Oh I., Kwon and Lee S. (1996) Purification andcharacterization of a fibrinolytic enzyme produced from*Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. Appl. Environ. Microbiol., 62, 2482–2488.

[7] Alain B. VouidibioMbozo, Simon C. Kobawila, AmarachukwuAnyogu, Brigitte Awamaria, DelphinLouembe, Jane P. Sutherland, L. Irene I. Ouoba. (2017).Investigation of the diversity and safety of the predominant Bacilluspumilussensulato and other Bacillus species involved in the alkalinefermentation of cassava leaves for the production of NtobaMbodi. *Food Control* 82 154-162.

[8] Louembé, D., S.C. Kobawila, G. BouangaKalou and S.Kéléké, (2003). Etude microbiologique des feuilles fermentées de manioc: “NtobaMbodi”. Tropicultura, 21 (3): 106-111.

[9] Kobawila, S.C., D. Louembe, S. Keleke, D.J. Hounhouigan and C. Gamba, (2005). Reduction of the cyanide content during fermentation of cassava roots and leaves to produce bikedi and ntobambodi, two food products from Congo. Afr. J. Biotechnol., 4 (7): 689-696.

[10] Ouoba LI[1], Parkouda C, Diawara B, Scotti C, Varnam AH. (2008). Identification of Bacillus spp. from Bikalga, fermented seeds of Hibiscus sabdariffa: phenotypic and genotypic characterization.ApplMicrobiol. Jan;104 (1):122-31. Epub 2007 Oct 8

[11] Ahaotu EO, Umoh G, Onwueagba AE, Chukwu AO, Iwuanyanwu UP (2013). Guinea Fowl Keets Performance Under Improved AndExtensive Conditions In Anthony Patience Farms, Atta – Ikeduru, Imo – State, Nigeria. Inter. J. Agri. Biosci., 2 (2): 82-86.

[12] Parkouda C, Nielsen DS, Azokpota P, Ouoba LII, Amoa-Awua WK, Thorsen L, Hounhouigan JD, Jensen JS, Tano-Debrah K, Diawara B, Jakobsen M (2009). The microbiology of alkaline-fermentation ofindigenous seeds used as food condiments in Africa and Asia. Crit. Rev. Microbiol. 35: 139-156.

[13] Etienne Nguimbi, Gabriel Ahombo, Rachel Moyen, Raoul Ampa, Alain Vouidibio, Esther, Nina Ontsira, Simon Charles, Kobawila and DelphinLouembe **(**2014**).**Optimization of Growth, Fibrinolytic Enzyme Production and PCR Amplification of Encoding Fibrinolytic Enzyme Gene in *Bacillus amyloliquefaciens*isolated from Ntobambodi at Brazzaville. *International Journal of Science and Research (IJSR)*, 3 (11): 2799-2803.

[14] Puri S, Beg QK, Gupta R. (2002). Optimization of alkaline protease production from Bacillus sp. *by response surface methodology CurrMicrobiol*, 44: 286-290.

[15] SolokaMabikaArmelFaly, Rachel Moyen, Etienne Nguimbi*, Gabriel Ahombo, Raoul Ampa, Aimé Christian Kayath, Alain Vouidibio, Cyr Jonas Morabandza and Simon Charles Kobawila (2017). Production, Partial Purification and Based SDS-PAGE Profiles of Caseinolytic Enzyme in two Bacillus Strains Isolated from Fermented Cassava leaves "Ntobambodi" in Congo Brazzaville. *Journal of Pure and Applied Microbiology*. March. Vol. 11 (1), p. 77-86

[16] Astrup T., Mullertz S. (1952).The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.*, ;**40**, 346-351.

[17] Kim S. and Choi N.S (1999). Electrophoresis analysis of protease inhibitors in fibrin zygmography.*Anal.Bochem.*, ;**270**: 179- 181.

[18] Wang S.H., Zhang C., Yang Y.L., Diao M., Bai M.F. (2007). Screening of high fibrinolytic enzyme producing strain and characterization of the fibrinolytic enzyme produced from Bacillus subtilis LD 8547. World J. Microbiol. Biotechnol., 7:epub.

[19] Kim, Gyoung Min, Ae Ran Lee, Kang Wook Lee, Jae-Yong Park, Jiyeon Chun, Jaeho Cha4, Young-Sun Song5, and Jeong Hwan Kim1* (2009).Characterization of a 27 kDaFibrinolytic Enzyme from Bacillus amyloliquefaciens CH51 Isolated from Cheonggukjan J. Microbiol. Biotechnol. 19 (9), 997–1004

[20] Yong Peng 1, Xiao-Juan Yang, Lu Xiao, Yi-Zheng Zhang ∗ (2004).Cloning and expression of a fibrinolytic enzyme (subtilisin DFE) gene from *Bacillus amyloliquefaciens*DC-4 in *Bacillus subtilis.* Research in Microbiology 155 167–173.

[21] wonkeukkim, keehyunchoi, yongtaekkim, hyunghwan park, jangyoonchoi, yoonsoo lee, hoonil oh, ikboo kwon, and shinyoung lee. (1996). Purification and Characterization of a fibrinolytic enzyme produced from bacillus sp. strain ck 11-4 screened from chungkook-jang. Applied and environmental microbiology, p. 2482–2488 vol. 62.

[22] Wang S.-H., Diao M., Yang Y.-L., Lin W.-Z., Huang B.-F (2008). Gene clone and expression of a fibrinolyticenzyme (FE) in *Escherichia coli.*Annals of Microbiology, 58 (1) 95-98).

[23] Stothard P (2000) The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques 28:1102-1104

[24] Lee, S. K., S. Heo, D. H. Bae, and K. H. Choi. (1998). Medium optimization for fibrinolytic enzyme prodution by*Bacillus subtilis*KCK-7 isolated from Korean traditional Chungkookjang. *Kor. J. Appl. Microbiol. Biotechnol*. **26:**226-231.
[25] Yoon, Seon-Joo, Myeong-Ae Yu, Gwan-Sub Sim, Seung-Taek Kwon, Jae-Kwan Hwang, Jung-Kue Shin, Ik-Hyun Yeo, and Yu-Rang Pyun (2002), Screening and Characterization of Microorganisms with Fibrinolytic Activity from Fermented Foods, *J. Microbiol. Biotechnol*. 12 (4), 649–656.
[26] Sumi, H. (1990). Development of nattokinase and healthy natto (in Japanese). *Bioindustry***7:** 725-731.
[27] PonnuswamyVijayaraghavan and Samuel Gnana Prakash Vincent (2014). Statistical Optimization of Fibrinolytic Enzyme Production Using Agroresidues by *Bacillus cereus* IND1 and Its Thrombolytic Activity *In Vitro* Hindawi Publishing Corporation BioMed Research International Volume, 11 pages
[28] Anjana Sharma*, Arunima Sharma and L. Shivlata (2015). Optimization of Medium Components for Enhanced Production of Extracellular Fibrinolytic Protease from *Citrobacter braakiiInt. J.Curr.Microbiol.App.Sci*4 (7): 248-259
[29] Ji-Hyun Seo and Sam-Pin Lee (2004).Production of Fibrinolytic Enzyme from Soybean Grits Fermented by Bacillus firmus NA-1 Article in Journal of Medicinal Food 7 (4):442-9
[30] Peng Y, Huang Q, Zhang RH, Zhang YZ (2003) Purification and characterization of a fibrinolytic enzyme produced by Bacillus amyloliquefaciens DC-4 screened from douchi, a traditional Chinese soybean food. Comp BiochemPhysiolBiochemMolBiol 134:45–52.
[31] Jeong YK, Park JU, Baek H, Park SH, Kong IS, Kim DW, Joo WH (2001) Purification and biochemical characterization of a fibrinolytic enzyme from Bacillus subtilis BK-17. World JMicrobiolBiotechnol 17:89–92.
[32] Ko JH, Yan JP, Zhu L, Qi YP (2004) Identification of two novelfibrinolytic enzymes fromBacillus subtilisQK02. Comp BiochemPhysiol C ToxicolPharmacol 137:65–74