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

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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 NM41 and 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 exhibits 99% of similarity with subtilisin gene of B.subtilis GI, accession number EF061457.1 in GenBank. While that encoding fibrinolitic enzyme in B. licheniformis NM74 exists 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 in thrombolysis 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-4 from 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 to ferment cassava leaves, as the process eliminates or decreases significantly the presence of toxic compounds 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 rise of 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. pumilus, 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 same conditions enzyme production vary from one strain to another [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.

Table 1: Primers used in the present study.

<table>
<thead>
<tr>
<th>No</th>
<th>Primer sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set1</td>
<td>P1: 5'-GGATCCCAAGAGGCTGCTGTGAC-3'F</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P2: 5'-GAATTCCTCGAGGATCCCGCTGATCA-3'R</td>
<td></td>
</tr>
<tr>
<td>Set2</td>
<td>P3: 5'-TGATACGACGTTCCTGCCTGTC-3'F</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P4: 5'-AAATTGTGAGATTAAATATGAA-3'R</td>
<td></td>
</tr>
<tr>
<td>Set3</td>
<td>P5: 5'-CATCACCAGGAAAAGAAGGTTTTGTC-3'F</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P6: 5'-CATCACCAGGAAAAGAAGGTTTTGTC-3'R</td>
<td></td>
</tr>
</tbody>
</table>

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 1). PCR was performed using a GeneAmp 2400 PCR system (Perkin Elmer, Watham, 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

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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 1. 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 held with SMS-ORF finder (National Center for Biotechnology). Sequences alignment were performed with Clustal and MUSCLE.

To make sequences analysis, we firstly used BLaSt 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](image1.png)

**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* and *Bacillus megaterium* and less important in *Bacillus pumilus.*

By anyway growth is observed in all strains.

![Figure 1b](image2.png)

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

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 (NM28 and NM29) 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 3: 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, Band C the annealing temperature is 58°C.](image)

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

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. Subtilisin 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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Nucleotides sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.l.NM74</td>
<td>GAT GGC ATT CGA CTC GGC</td>
</tr>
<tr>
<td>B.s.NM41</td>
<td>GCT TGT TGT CGT TAC GTT</td>
</tr>
<tr>
<td>B.s.NATT</td>
<td>GTA TGA AAA TAG TTA TTT CGA</td>
</tr>
<tr>
<td>Bsp. C4 SS-2013</td>
<td>CGA GTC TCT AGC GAA ATA CGC</td>
</tr>
<tr>
<td>B.s 50s</td>
<td>ATG AGA AGC AAA AAA TTG TGG</td>
</tr>
</tbody>
</table>

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 homologous, from the Protein Bank.

Figure 3, is confirming that, in the pre, the pro and the mature peptide B.1.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 proteoloytic 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. subtilis 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 – Fibrinolytic –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.


