

Optimization of Growth, Fibrinolytic Enzyme Production and PCR Amplification of Encoding Fibrinolytic Enzyme Gene in *Bacillus amyloliquefaciens* Isolated from Ntoba mbodi at Brazzaville

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Abstract: Two strains of *Bacillus amyloliquefaciens* screened from Ntoba Mbodi, respectively *B.a* NM76 and *B.a* NM 77 were tested as strong producers of a fibrinolytic enzyme. Monitoring of growth and enzyme production were assayed. Experiments were conducted about the influence of temperature and PH on the growth and fibrinolytic enzyme production. For Temperature, growth and enzyme production were observed: from 25°C to 50°C with the optimum temperature between 35°C to 40°C for growth, and the optimum between 30°C and 35°C for fibrinolytic enzyme production. For pH from 5 to 11, growth was possible with the optimum at pH 7, enzyme production was possible in the range of pH from 5 to 9, with the optimum at 8. The relationship between growth and enzyme production has shown that the later usually is optimal during the stationary phase of the growth. Moreover, PCR amplification of the encoding fibrinolytic enzymes genes in the two strains has been hold and the results has shown two identical specific bands, as illustrated by the agarose gel electrophoresis of the PCR products.

Keywords: Optimization, Fibrinolytic, PCR-amplification, Ntoba Mbodi, *Bacillus amyloliquefaciens*

1. Introduction

According to the report by world Health Organization (WHO) in 2001, 17 million people die of cardiovascular diseases (CVD) every year. The formation of a clot of blood in a blood vessel is one of the main causes of a variety of CVDs. The accumulation of fibrin in the blood vessels usually increases thrombosis, leading to myocardial infarction and other cardiovascular disease under abnormal conditions.[4]. Fibrin is formed from fibrinogen via the proteolysis by thrombin. Meanwhile, the fibrin clots can be hydrolyzed by plasmin to avoid thrombosis in blood vessels[12]. In recent years, thrombolytic therapy has been extensively researched as a means of medical treatment of the blood clot, and various fibrinolytic enzymes produced by different microorganisms were discovered [2, 7,16].

The genus *Bacillus* from traditional fermented food is an important one among the microorganisms that have been found to produce the fibrinolytic enzymes. *Bacillus natto* producing NK was the first screened from a traditional Japanese soybean-fermented food named natto[15]. Over the years, other bacilli have been discovered to produce fibrinolytic enzymes. they are *B. amyloliquefaciens* DC-4 from Chinese soybean-fermented food [13], *Bacillus* sp.CK from Korean fermented-soybean sauce [6]. Some have been isolated in other sources, the case of *Bacillus subtilis* EM 29 isolated from dried herb in China, with produce a strong fibrinolytic enzyme [11]. *B. amyloliquefaciens* CH51, previously isolated from cheonggukjang, showed higher fibrinolytic activity than other isolates when examined by the fibrin plate method. [8]

In the republic of Congo fermented manioc leaves are used as a food by Congolese in some area, they are differently called: ntoba mubodi in the Bouenza, Lilleyuka in the Kouilou or Ntoba mbodi in the Pool[10]. The microbiological study of Ntoba mbodi have been hold, it has shown the diversity of microorganisms, among them species of the genus *Bacillus* [10].

In this work two strains of *Bacillus amyloliquefaciens* NM76 and NM77 isolated from Ntoba Mbodi, and which produce a strong fibrinolytic enzymes were assayed. Monitoring of growth and enzyme production were hold, and the relationships between the two phenomens analyzed Moreover the encoding fibrinolytic enzymes genes were PCR amplified and electrophoretically analyzed.

2. Material and Methods

2.1 Bacterial Strains and culture conditions

E.coli K12 in the laboratory, tested as non producer of a fibrinolytic was used as a negative control. Other strains were isolated from Ntoba mbodi (NM), *Bacillus amyloliquefaciens* NM-76 and NM77 were used for experiments in this study. *Bacillus subtilis* NM 78 which produce fibrinolytic enzyme available in the Laboratory was used as a positive control. Strains were grown in Luria-Bertani (LB) broth for 48 h at different temperatures with shaking. After optimization of culture conditions, the cultures were hold at 37°C. The pH was adjusted at 7.

2.2 Fibrinolytic enzyme assay

Bacillus cells were cultivated in LB at 37°C with shaking. At the appropriate growth stage, the culture was centrifuged at 4000t/minute for 10 minutes at 4°C. The supernatant was assayed for fibrinolytic activity. Fibrinolytic activity was determined using the fibrin plate method with modification [1, 9, 18, 16, 17]. 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.

2.3 Influence of temperature on growth and enzyme production

Culture was hold on LB at different temperatures from 25 to 50°C during 48 hours. Each two hours 1ml of culture was used to measure growth through the optical density value (OD) at 600nm with a spectrophotometer , 1ml of culture was centrifuged, and the supernatant used for the fibrinolytic activity on fibrin plate. Each experiment was hold twice.

2.4 Influence of pH on the growth and enzyme production

Culture was hold on LB at different pH from 5 to 10 during 48 hours. Each two hours 1ml of culture was used to measure growth through the optical density value (OD) at 600nm with a spectrophotometer , 1ml of culture was

centrifuged, and the supernatant used for the fibrinolytic activity. Each experiment was hold twice

2.5 PCR amplification of encoding fibrinolytic enzyme genes in the two strains

Genomic DNA of the two strains was isolated and used as a template [17]. To amplify DNA fragments encoding fibrinolytic enzyme genes in the two strains, optimization was made using two sets of primers according to the literature (Table 1). PCR was performed using a GeneAmp 2400 PCR system(PerkinElmer, 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 optimized for each pair 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, 62°C, 64°C) for 30 sec., and extension at 72°C for 40sec., final extension at 72°C for 5 min.

3. Results

3.1 Fibrinolytic Activity

Two strains of *Bacillus amyloliquefaciens* have shown on fibrin plate a strong fibrinolytic activity **Figure 1**.

3.2 Influence of temperature on growth and extracellular fibrinolytic enzyme production

Growth profiles of B.aNM76 and B.a NM77at 37°C are identical; it is shown in **Figure 2**. It shows four characteristic from 0 to 2 hours the latency phase, from 2 to 10 hours the exponential phase, from 10 to 28 hours the stationary phase and after 28 hours the decreasing phase

Figure 3 represents the variation the growth profile at different temperatures specially the amplitude variation from 25 to 50 °C. It is shown the growth profile is proportional to the increase of the temperature from 25°C to 37°C, and inversely between from 37°C to 50°C The optimal temperature is around 37°C.

Extracellular fibrinolytic enzyme production is shown in **Figure 4**. This figure is showing the fibrinolytic enzyme production at different temperatures, each graph comprise a latency phase from 0 to 6 hours , a exponential phase from 6 to 28 hours, after 28 hours a decrease phase .

The amplitudes of different graphs are proportional to the increasing of temperature from 25°C to 37 °C, after 37°C amplitudes are inversely proportional to the increase of the temperature. At 50 °C there is no enzyme production.

The most important is that all temperatures the optimal production of the enzyme is during the stationary phase of the growth

Influence of PH on growth and extracellular fibrinolytic enzyme production

Figure 5 shows that growth is possible from pH 5 to 11, with the optimum at pH 7. **Figure 6** shows the variation of extracellular fibrinolytic enzyme production, which is only possible from pH 5 to 9, with the optimum at pH 7.

Electrophoresis analysis of PCR amplified encoding fibrinolytic enzyme gene.

Figure 7 shows the Agarose gel electrophoresis of PCR amplified encoding fibrinolytic enzyme gene. According to **Figure 7**, for the primers set1, in all three annealing temperatures are displaying no specific bands. For the primers set2, it is obvious that the optimal conditions of the PCR amplification of encoding fibrinolytic enzyme genes in the two strains of *Bacillus amyloliquefaciens* NM76 et NM77 are those with the annealing temperature 58. For the primers set2, the negative control displays no band, otherwise the other three strains displays specific bands. The two strains of *Bacillus amyloliquefaciens* NM-76 and NM-77 have given an identical band with same size. *Bacillus subtilis* displays a specific band with a particular size.

4. Discussion

The temperature is an important factor for growth, the growth profile of *B.a* NM76 and *B.a* NM 77 are almost identical to the general profile of bacterial growth, and this result is the same than which was published [5]

Growth and extracellular fibrinolytic enzyme production are two distinct phenomena as testify **Figure 3** and **Figure 4**. For all temperatures enzyme production is almost maximum during the stationary phase of the growth, before six hours when the growth have already started, the enzyme production is absent or small. These results are similar than which was published [3, 5, 11].

Growth is observed at all pH from 5 to 11, with the optimum at pH 7, but enzyme production is not observed in pH 11. During growth bacteria produce many products including the fibrinolytic enzyme. pH 11 may be too basic for these strains. During the first stage of growth the absence of enzyme activity doesn't always mean the absence of enzyme production, this hypothesis will be confirmed after the purification of the enzyme. The enzyme is a protein, transcription, translation or post transcriptional mechanism can be influenced, and all those aspects may influence enzyme production. Culture conditions for the production of fibrinolytic enzymes must be chosen carefully since they may affect the activity of the enzyme.

Agarose gel electrophoresis of PCR amplification of encoding fibrinolytic genes products has shown the optimal conditions for the strains of *Bacillus amyloliquefaciens* (*B.a* NM-76 and *B.a* NM- 77) at annealing temperature 58°C for the primers set2(**Figure7**). This figure is showing clearly two identical specific bands for the two strains, the size of the two bands according to the standard marker is around 1.8kb. These results are in concordance with those already published [14]. Moreover the length of the positive control amplified fragment is around 1.3kb, this result is similar than which was already got [8].

Kim et al. 2004 have demonstrated that a strain may have more than one fibrinolytic enzyme which may have different sizes. For the same sets of primers can be used to amplify the fibrinolytic enzyme genes of different sizes in different strains of *Bacillus*, these strains must have conserved regions which allow the primers to anneal.

5. Conclusion

Optimization of culture conditions through the influence of temperature and pH has allowed us to know the optimum conditions of growth and enzyme production by *Bacillus amyloliquefaciens* isolated from Ntoba mbodi. PCR-amplification conditions are well known and will be very helpful because sequencing the fibrinolytic enzyme gene fragment will be the next step.

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Table 1: Sets of Primers Used in this Study

Primers set 1	5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC -3' F 5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3' R	[8]
Primers set 2	5'-TCACAGCTTTTCTCGGTC-3' F 5'-TGATCCGATTACGAATGC -3' R	[14]

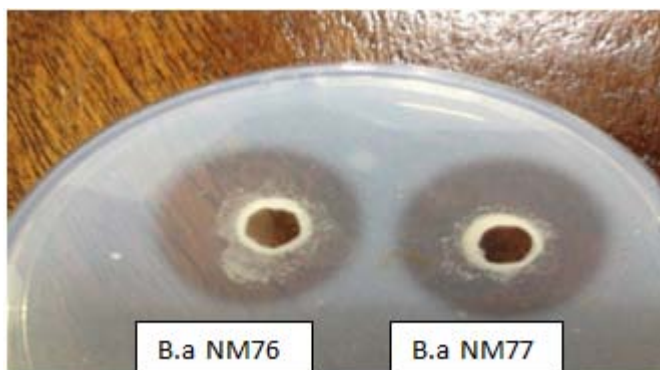


Figure 1: halo of fibrinolytic activity of B.a NM76 and B.a NM77 on a fibrin plate

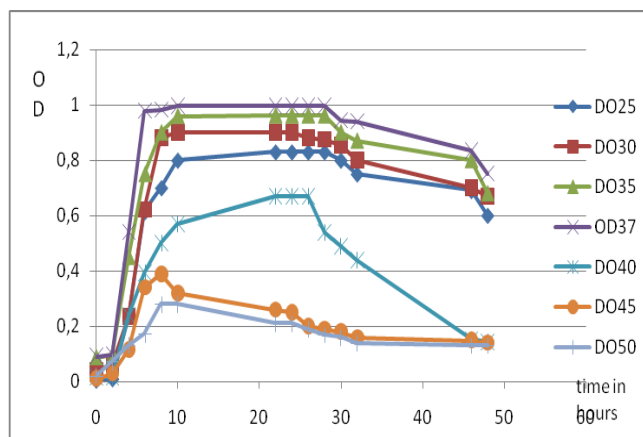


Figure 3: Graph of growth profiles at different temperatures (25°C-50°C)

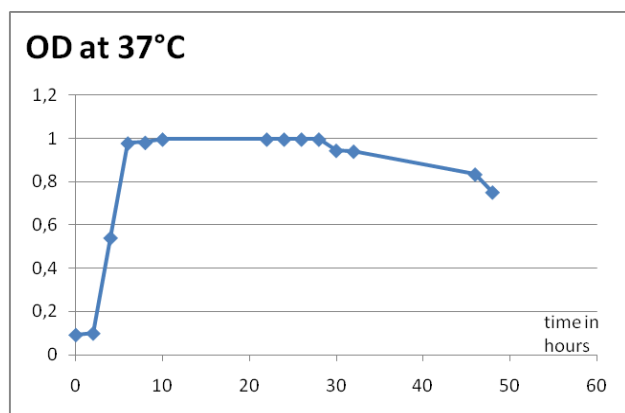


Figure 2: Growth profile of B.amyloliquefaciens NM 76 or MN77 at 37°C

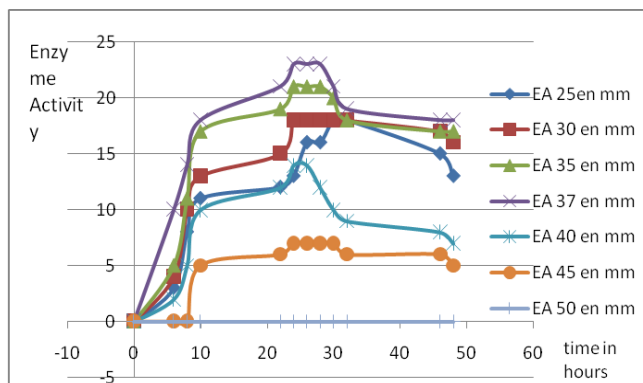


Figure 4: Profiles of fibrinolytic enzyme activity at different temperatures

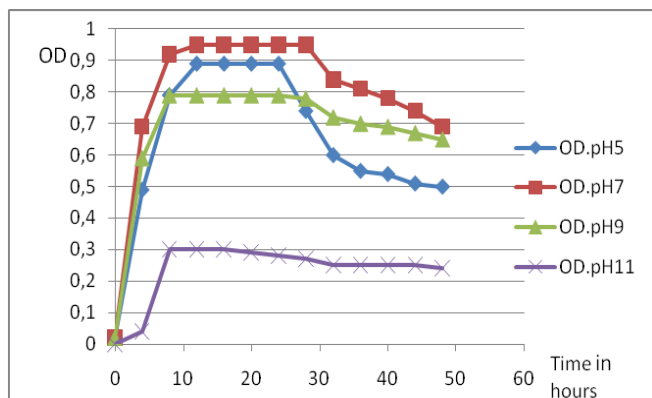


Figure 5: Graph of growth profiles at different pH

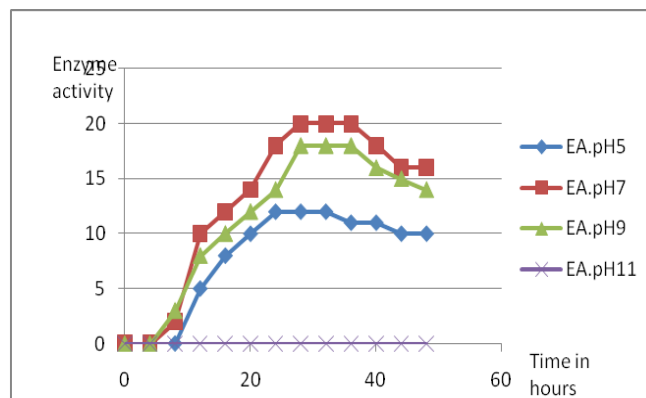
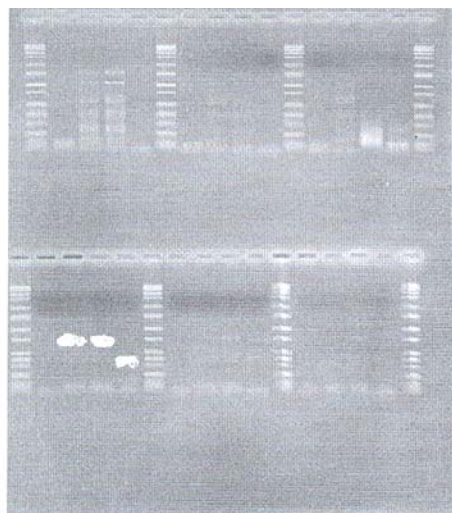


Figure 6: Profiles of fibrinolytic enzyme activity at different PH



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 7 :Gel electrophoresis of encoding fibrinolytic enzyme genes PCR amplified products from 4 strains in different conditions.

primers set1

1,2,3,4,5,6: Standard (S), E.coli K12, NM76, NM77, NM78, standard: annealing temperature 58;

7,8,9,10,11: Ecoli K12, NM76, NM77, NM78, standard: annealing temperature: 62;

12,13,14,15,16: E.coli K12, NM76, NM77, NM78, standard: annealing temperature 64.

primers set2

1,2,3,4,5,6: Standard, E.coli K12, NM76, NM77, NM78, standard: annealing temperature 58

7,8,9,10,11: E.coli K12, NM76, NM77, NM78, standard: annealing temperature: 62;

12,13,14,15,16: E.coliK12, NM76, NM77, NM78, standard: annealing temperature 64.

Table 1: sets of primers used in this study

Figure 1: Halo of fibrinolytic activity of B.a NM76 and B.a NM77 on a fibrin plate

Figure 2 : Growth profile of B.amyloliquefaciens NM 76 or MN77 at 37°C

Figure 3: Graph of growth profiles at different temperatures (25°C-50°C)

Figure 4: Profiles of fibrinolytic enzyme activity at different temperatures

Figure 5: Graph of growth profiles at different pH

Figure 6: Profiles of fibrinolytic enzyme activity at different pH

Figure 7: Gel electrophoresis of encoding fibrinolytic enzyme genes PCR amplified products from 4 strains in different conditions