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 NM77are 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 trains of *Bacillus amyloliquefaciens* NM-76 and NM-77 have given an identical band whith 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 to 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, traduction or post transcriptional mechanism can be influenced, and all those aspect may influence enzyme production. Culture conditions for the production of fibrilolytic 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 that 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 know and will be very helpful because sequencing the fibrinolytic enzyme gene fragment will be the next step.

6. Acknowledgements

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References

[1] Astrup T., Mullertz S. 1952. The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. Biophys., 40, 346-351.
[2] Choi H S, Shin HH. 1998. Purification and partial characterization of a fibrinolytic protease in Pleurotus ostreatusMycologia ;90:674–9.
[3] Choi N. S., Chung D.M., Ryu C.H, K.-S. Yong P., Maeng J, and Kim S. H. 2006. Identification of three extracellular proteases from Bacillus subtilis KCTC 3014. J Microbiol. Biotechnol.,. 16: 457-464.
[4] Hwang K.-J., Choi K.-H., Kim M.-J., Park Ch.-S. and Cha J.*2007. Purification and Characterization of a New Fibrinolytic Enzyme of Bacillus licheniformis KJ-31, Isolated from Korean Traditional Jeot-gal. J. Microbiol. 17(9), 1469-1476
[5] kannan N., Aravindan R. and Viruthagiri T. 2011. Effect of culture conditions and kinetic studies on extracellular tannase production by *Lactobacillus plantarum*MTCC1407.). Indian Journal of Biotechnology, Vol 10, pp321-328
[6] Kim, W., Choi, K., Kim, Y., Park, H., Choi, J., Lee, Y**.,** Lee H., Oh I.,Kwon and Lee S. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. Appl. Environ. Microbiol., 62, 2482–2488.
[7] Kim, H., Kim G., and Kim D. 1997. Purification and characterization of a novel fibrinolytic enzyme from Bacillus sp. KA38 originated from fermented fish. J. Ferment. Bioeng., 84: 307-312
[8] 8-Kim G. M., Lee A. R., Lee K. W., Park J.-Y., Chun J., Cha J., Song Y-S and Kim J. H. 2009. Characterization of a 27 kDa Fibrinolytic Enzyme from Bacillus amyloliquefaciens CH51 Isolated from Cheonggukjang. J. Microbiol. Biotechnol., 19(9), 997–1004

[9] Kim S. and Choi N.S. 1999. Electrophoresis analysis of protease inhibitors in fibrin zygmography.Anal. Bochem., 270 :179-181
[10] Louembé D., Kobawila S.C, Bouanga Kalou G. and Kéléké S. 2003. Etude microbiologique des feuilles fermentées de manioc ‘’Ntoba mbodi’’. Tropicultura,. Vol21, N°3 ; 106-111
[11] Nguimbi E., Wu Z.R. 2002.Production of a New Fibrinolytic Enzyme, Bacterium Growth and Enzyme Production Conditions, Purification and characterization of the New Enzyme. () Biotechnology, Vol. 12, N° 2.
[12] Peng Y., Yang X., Zhang Y. 2005.Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo. Appl. Microbiol. Biotechnol., 69: 126–132
[13] Peng Y., Zhang Y.Z. 2002. Isolation and characterization of fibrinolytic enzyme-producing strain DC-4 from Chinese douchi and primary analysis of the enzyme property, High Technol. Lett., 12 (2) 30–33
[14] Peng Y., Yang X-J., Xiao L., Zhang Y.-Z. 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
[15] 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.
[16] 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.
[17] Wang S.-H., Diao M., Yang Y.-L., Lin W.-Z., Huang B.-F. Gene clone and expression of a fibrinolytic enzyme (FE) in *Escherichia coli* .Annals of Microbiology2008, 58 (1) 95-98)
[18] Zhang R.H., Xiao L., Peng Y., Wang H.Y., Bai F., Zhang Y.Z. 2005. Gene expression and characteristics of a novel fibrinolytic enzyme (subtilisin DFE) in Escherichia coli. Lett. App. Microbial., 41: 190-195.

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]

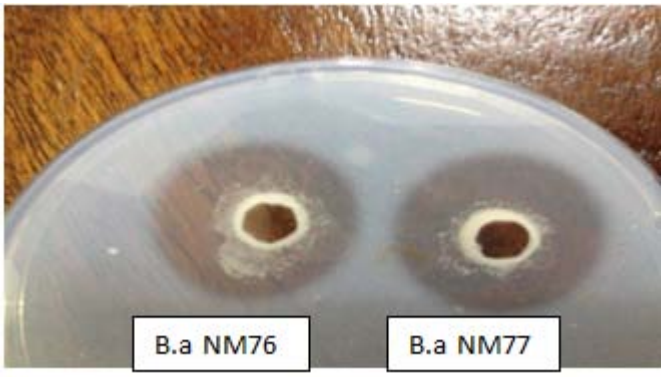

Figure 1: halo of fibrinolytic activity of B.a NM76 and B.a NM77 on a fibrin plate

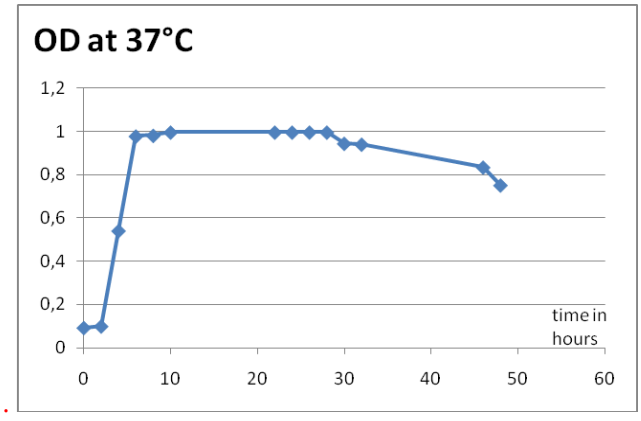

Figure 2: Growth profile of B.amyloliquefaciens NM 76 or MN77 at 37°C

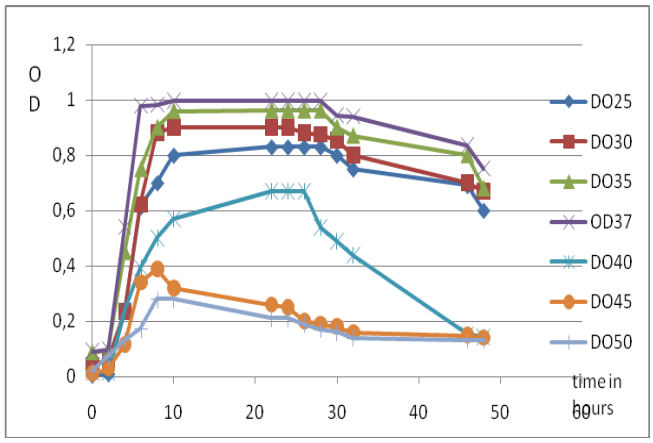

Figure 3: Graph of growth profiles at different temperatures (25°C-50°C)

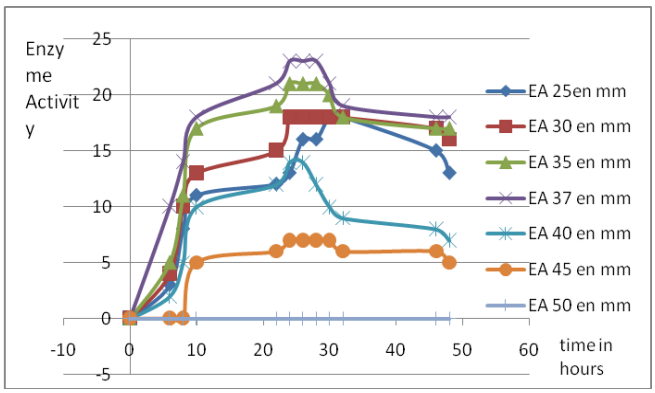

Figure 4: Profiles of fibrinolytic enzyme activity at different temperatures

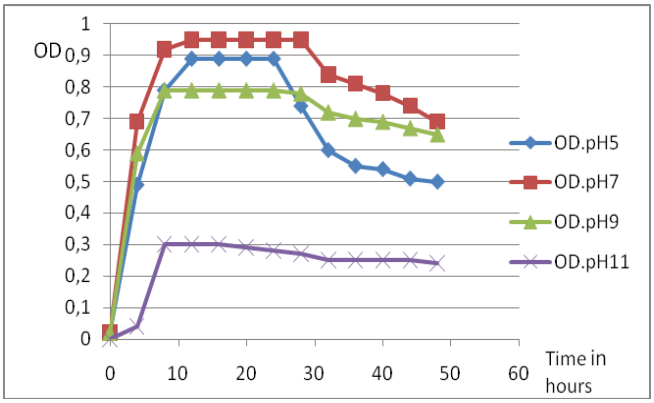

Figure 5: Graph of growth profiles at different pH

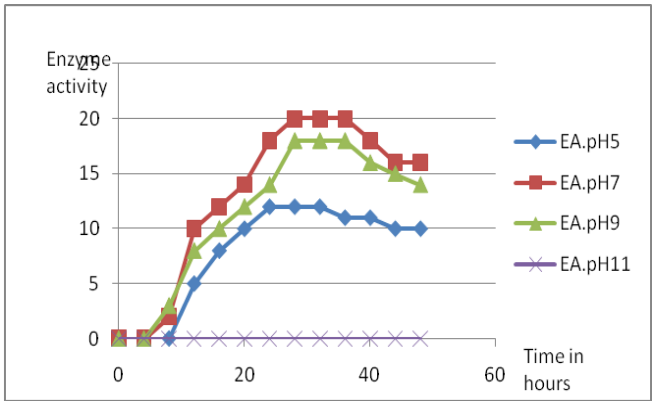

Figure 6: Profiles of fibrinolytic enzyme activity at different PH

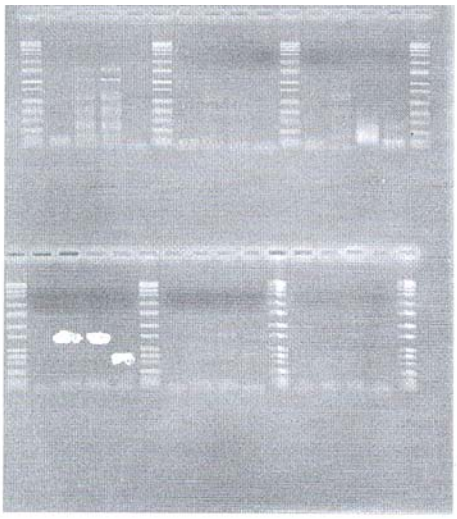

Figure 7 :Gel electrophoresis of encoding fibrinolytic enzyme genes PCR amplified products from 4 strains in different conditions.
primers set1
1,2,3,4,5,6: Standard (S), E.coli K12, NM76, NM77, NM78, standard: annealing temperature 58;
7,8,9,10,11: Ecoli K12, NM76, NM77, NM78, standard: annealing temperature: 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