

Production, Partial Purification and Based SDS-PAGE Profiles of Caseinolytic Enzyme in two *Bacillus* Strains Isolated from Fermented Cassava leaves "*Ntoba mbodi*" in Congo Brazzaville

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Two *Bacillus* strains isolated from *Ntoba mbodi*: *Bacillus megaterium* (*B.me* NM 02) *Bacillus licheniformis* (*B.li* NM01), has shown a significant caseinolytic enzyme activity. We set optimization of growth and enzyme production conditions. Several parameters have been optimized: temperature, pH, various types of media, carbon and nitrogen sources. In both strains, growth is possible from 25 to 60 °C with an optimum temperature at 37°C for *B.li* and at 35°C for *B.me*. Enzyme production was observed from 25 to 55 °C with an optimum temperature of 30 °C for *B.li*. Enzyme production was observed from 25 to 50 °C with an optimum temperature of 35 °C in *B.me*. For pH, growth and enzyme production can be at 5, 7 and 9 with an optimum at 7 for both strains. The LB medium is better for growth and enzyme production than TSB for *B.li* and *B.me*. Among the carbon sources used, fructose is better for growth after 48 hours of incubation in both strains (*B.li*: 0,93±0,001, *B.me*: 0,928±0,002), but for enzyme production fructose remains the best carbon source for *B.li* (14,33±1,24), while starch is the best for *B.me* (14,66±1,24). Concerning nitrogen sources, in both strains the best source of growth is the yeast extracts (*B.li*: 0,969±0,015, *B.me*: 0,952±0,01). For enzyme production, the two sources can be used for *B.li* (14,333±1,247) but for *B.me* (14,333±0,471) only the yeast extracts is the best as well as for growth and enzyme production. Furthermore, in both strains the produced enzyme was partially purified by using ammonium sulfate precipitation, and SDS-PAGE has been hold, profiles of specific bands are useful to give more information and differentiate the two strains.

Keywords: Optimization, caseinolytic enzyme, *Ntoba mbodi*, *Bacillus licheniformis*, *Bacillus megaterium*.

Enzymes are macromolecules involved in many cellular reactions. Proteases are groups of proteins included in the subclass hydrolases, within

the main class enzymes. Serine alkaline proteases (SAP) are one of the most important groups of industrial enzymes. They account for approximately 35% of the total microbial enzyme sales¹.

Proteases or proteolytic enzymes find many applications in many domains as : pharmacy, food, biotechnology, and detergents^{2, 3}.

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Commercially used proteases are produced by bacteria, plants, animals and fungi^{4, 5}. Proteases are produced by many groups of bacteria: *Bacillus*, *Pseudomonas* and others. The genus *Bacillus* is recognized as one of the most important producers of exocellular proteases^{1,5}. Protease production by micro-organisms is a natural phenomenon. *Bacillus* species produce proteases during the exponential and stationary phases during growth. Extracellular protease production is strongly influenced by media composition, the ratio of C/N, metabolizable sugars⁶ and metallic ions^{1,7}. Moreover, protease production is also affected by rapidly metabolizable sources, like amino acids in the medium and by some physical factors (aeration, inoculum density, pH and temperature). Protease production by microorganisms is connected to the growth and that is characteristic of each microorganism¹. Many studies have been focused on protease production by microorganisms, these studies have stated strategies of growth and protease production^{7, 8, 9}. Some studies have given information in the relation between growth and protease production. Those studies corroborate with the hypothesis that: protease production is controlled by number of mechanisms that occur in the transition between the end of the exponential phase and the beginning of the stationary phase^{10, 11, 12}. By any way all studies are strict about the variability and make an emphasis on the optimal conditions of protease production that depend strictly on each microorganism^{13, 14, 15}.

Caseinolytic proteases P(ClpPs) are complex of oligomeric proteins with contribute on cellular homeostasis and virulence regulation to bacteria. Whatever most of organisms possess only one ClpP protein, in some can be encoded two or more ClpP isoforms¹⁶. The caseinolytic protease P(ClpP) is a highly conserved enzyme in bacteria and higher organisms^{17, 18, 19}.

In the republic of Congo fermented manioc leaves are used as a food by Congolese in some area. They are differently called: Ntoba mbodi in the Bouenza, Lilleyuka in the Kouilou and Ntoba mbodi in the Pool. Microbiological study of the Ntoba Mbodi has been held and has shown a diversity of bacteria, among them the species of genus *Bacillus*²⁰.

Many species of genus *Bacillus* isolated from Ntoba Mbodi has been molecularly

characterized by their ARNr 16S and ARNr 16S-23S. Many of them have shown proteolytic activities, as the fibrinolytic enzyme producer *Ba* NM76 described in¹⁵. A new strain named *Lysinibacillus louembei* sp. with a strong proteolytic activity has been isolated from Ntoba Mbodi.²¹.

Two *Bacillus* strains isolated from Ntoba mbodi: *Bacillus megaterium* (*B.me* NM 02) *Bacillus licheniformis* (*B.li* NM01), have shown a strong caseinolytic enzyme activity. In the present work we optimize growth and enzyme production conditions (temperature, pH, nitrogen and carbon sources), produce the enzyme. In both strains the produced enzyme was partially purified by using ammonium sulfate precipitation. SDS-PAGE has been held and profiles used to differentiate the strains.

MATERIALS AND METHODS

Bacterial strains and media

Strains used in this study were *Bacillus megaterium* (*B.me* NM 02) and *Bacillus licheniformis* (*B.li* NM 01) available in our laboratory were isolated from Ntoba mbodi.

The media used were: Luria Bertani (0,5% NaCl, 1% Tryptone, 0,5% Extraits de levure); Tryptone soya broth (TSB); Amidon (0, 5%); Galactose (0, 5%); Fructose (0, 5%); and mineral solution (MgSO₄.5H₂O: 0,06%, KH₂PO₄: 0,1%, CaCl₂.6H₂O: 0,2%, K₂HPO₄: 0,2%).

Culture conditions

250ml Erlenmeyer containing 100ml of LB was inoculated with 1ml of overnight culture and incubated at 37°C for 48 hours. 2ml of bacterial suspension were used to compute the optical density (OD) at 600 nm with a type vis spectrophotometer (722 Ningb SI Instrument CO, LTD).^{12,15}

Enzyme assay

Puri modified techniques [9]. was used to test caseinolytic enzyme. The 48h culture was collected in 1,5 eppendorf and centrifuged for 5 minutes at 6000rpm in a type microcentrifuge(). The supernatant was collected for enzyme activity.

1g of agarose was dissolved in 250 ml erlenmeyer containing 100 ml de PBS at 0,1N, after boiling, we waited until à 55-60°C, 10 ml of skimmed milk were added. The mixture were poured in petri

dishes. Wholes were made on the plate. 50% 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^{22, 23}.

Influence of temperature on the growth and caseinolytic enzyme production

To study the effect of temperature on growth and caseinolytic enzyme production, we made culture on LB medium at different temperatures (25, 30, 35, 37, 40, 45, 50, 55 et 60°C) for 48 h. Each two hours optical density at 600nm and enzyme activity were computed. Testing caseinolytic activity plates were incubated at 37°C, for 12 h. Lytic area through the diameter of the clear zone was measured to show the enzyme activity^{12, 15, 22, 23}.

Influence of pH on the growth and caseinolytic enzyme production

To study the effect of pH on growth and caseinolytic enzyme production, we made culture on LB medium at different pH : 5, 7, 9 for 48 h. Each two hours optical density at 600nm and enzyme activity were computed. Testing caseinolytic activity plates were incubated at 37°C, for 12 h. Lytic area through the diameter of the clear zone was measured to show the enzyme activity^{15, 22}.

Influence of different kinds of media on the growth and enzyme production

Two kinds of media were used to study their effects of on growth and caseinolytic enzyme production, we made culture on LB and TSB at pH : 7 for 48 h. Each two hours optical density at 600nm and enzyme activity were computed. Testing caseinolytic activity plates were incubated at 37°C, for 12 h. Lytic area through the diameter of the clear zone was measured to show the enzyme activity^{22, 23, 24}.

Influence of carbon sources on the growth and caseinolytic enzyme production

Three kinds of carbon sources (galactose, Fructose, amidon) were used to study their effects on growth and caseinolytic enzyme production, we made culture on three different media which compositions are given in **table 1**. Each two hours optical density at 600nm and enzyme activity were computed. Testing caseinolytic activity plates were incubated at 37°C, for 12 h. Lytic area through the diameter of the clear zone was measured to show the enzyme activity^{23, 25, 26}.

Influence of nitrogen sources on the growth and caseinolytic enzyme production

Two kinds of nitrogen sources (yeast extracts, Bactopeptone) were used to study their effects on growth and caseinolytic enzyme production, we made culture on two different media which compositions are given in **table 2**. Each two hours optical density at 600nm and enzyme activity were computed. Testing caseinolytic activity plates were incubated at 37°C, for 12 h. Lytic area through the diameter of the clear zone was measured to show the enzyme activity^{23, 23, 26}.

Proteins SDS-PAGE

Protein SDS- PAGE has been hold. A 48h culture was centrifuged and the supernatant was precipitated with ammonium sulfate. The centrifugation of the solution was at 4°C, 10000t/min for 1hour with the centrifuge type MICRO STAR 17R from VWR..The precipitate was diluted in PBS and 100 µl was used as a sample

The reagents were: Acrylamids 30% / biacrylamids 0.8 % ; Tris-HCL 1.5 M pH 8.8 ; Tris-HCL 0.5 M pH 6.8 ; Electrophoresis buffer : Tris 25 mM, Glycine 0.192 M, SDS 0.1% ; Staining solution : Coomassi bleu 2.5 g, méthanol 450 ml, glacial acétique acid 100 ml, eau 400 ml, ajusted at 1 l ; Dying solution : méthanol, glacial acétique acid 100 ml, eau 400 ml, ajusted at 1 l.; Protein molecular markers protéines (Protein Page Rulerprestained, Fermentas)

RESULTS

Caseinolytic enzyme activity

The Figure1 shows the caseinolytic activity in three bacillus strains isolated from Ntoba Mbodi : *B.me.*, *B.li.*, *B.amy.* The *B.amy.* is used as a positive control. The halo of *B.me* is more important than of the positive control. The halo of *B.li* is as important as of a positive control. The diameter of the halo is the expression of the enzyme production of the strain. *E.coli* is used as a negative control.

Influence of temperature on growth and caseinolytic enzyme production

Fig-2 shows the influence of temperature on growth profiles for *Bacillus licheniformis* with the time. For the temperatures from 25 to 40°C, the increase of temperature is proportional with growth profiles. In contrast, after 40°C the increase of temperature become inversely proportional to the

growth profiles. Growth is possible from 25 to 60°C, with the optimal temperature at 37°C.

All growth profiles have the same phases; exponentially phase, stationary phase and the decreasing phase, but the different amplitudes.



B.me : *Bacillus megaterium*, *B.li* : *Bacillus licheniformis*, *B.amy*: *Bacillus amyloliquefaciens*

Fig. 1. Halo of caseinolytic enzyme activity of different *Bacillus* strains isolated from Ntoba Mbodi

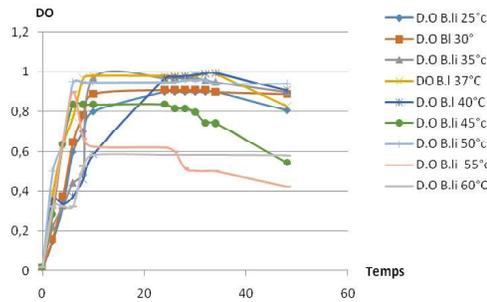


Fig. 2. Growth profiles of *Bacillus licheniformis* at different temperatures

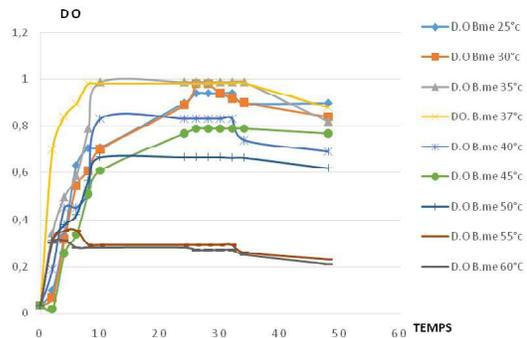
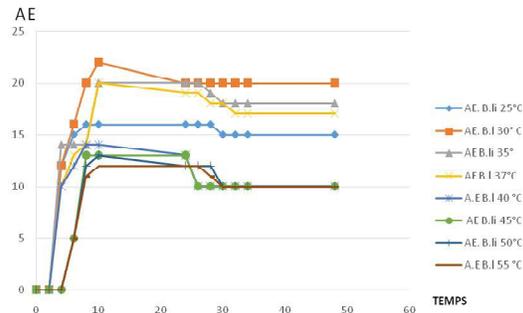


Fig. 4. Growth profiles of *Bacillus megaterium* at different temperatures

Fig-3 shows the influence of temperature on caseinolytic enzyme production for *Bacillus licheniformis*. Indeed, the enzyme activity here is used to express the enzyme production with the incubation time. For the temperatures from 25 to 30°C the amplitude of caseinolytic enzyme production profiles are proportional to increase of the temperature. For the temperatures from 30 to 55°C the amplitude caseinolytic enzyme production profiles is inversely proportional the increase of temperature. courbes de la production enzymatique augmentent, cela indique que la production enzymatique augmente. Caseinolytic enzyme production is possible for the temperature from 25 to 55°C, with optimal temperature of 30°C.

All profiles of caseinolytic enzyme production have the same phases; latency phase, exponentially phase of enzyme production,



AE=enzyme activity=testing the enzyme production

Fig. 3. Profiles of caseinolytic enzyme production by *Bacillus licheniformis* at different temperatures

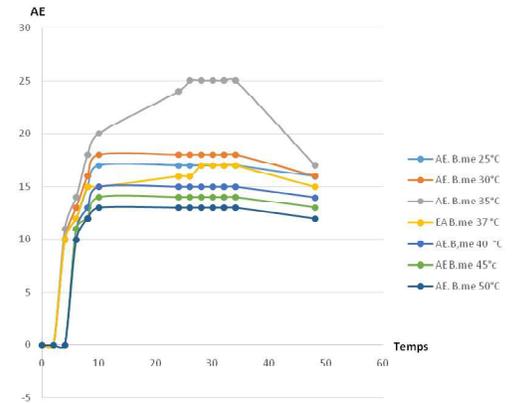


Fig. 5. Profiles of caseinolytic enzyme production for *Bacillus megaterium* at different temperatures

stationary phase of enzyme production and the decreasing phase, but the different amplitudes.

Fig-4 shows the influence of temperature on growth for *Bacillus megaterium*. For the temperatures from 25 to 45°C, the increase of temperature is proportional to the increase of the amplitudes of growth profiles. For the temperatures from 40 to 60°C, the increase of temperature is inversely proportional to the amplitudes of growth profiles. The growth of the strain is possible from 25 to 60°C, with the optimal temperature at [35°C-37°C]

All growth profiles have the same phases; exponentially phase, stationary phase and the decreasing phase, but the different amplitudes.

Fig-5 shows the influence of temperature on caseinolytic enzyme production for *Bacillus*

megaterium. For the temperature from 25 to 35°C, the increase of temperature is proportional to the increase of the amplitude of the enzyme production. For the temperature from 35 to 50°C, caseinolytic enzyme production decrease when the temperature increase. the caseinolytic enzyme production is possible from 25 to 50°C with the optimum at 35°C.

Both profiles have the same phases which are in correlation with the normal growth profile of bacteria. For both profiles from 0 to 10h, we have the exponentially phase. Stationary phase is from 10 to 30 hours, for *B.li*, while from 10 to 34 hours. The decrease phase is from 30 to 48 hours for *B.li*, from 34 to 48 hours for *B.me*.

Fig-7. shows different phases of the caseinolytic enzyme in the two strains. In the both strains, the latency phase is observed between 0 to 2h.

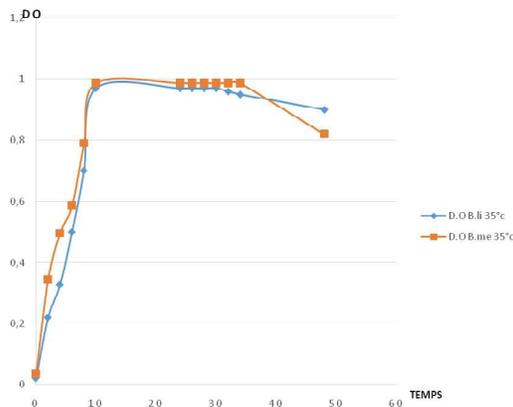


Fig. 6. Comparing growth profiles of *Bacillus licheniformis* (B.li) and *Bacillus megaterium* (B.me)

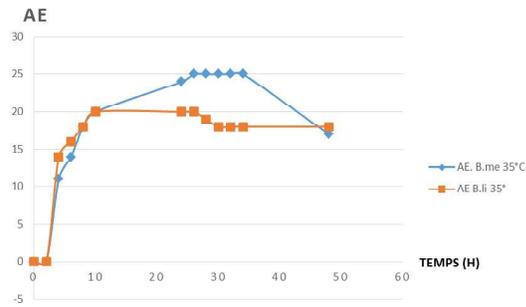


Fig. 7. Comparing profiles of caseinolytic enzyme production of *Bacillus licheniformis* (B.li) and *Bacillus megaterium* (B.me)

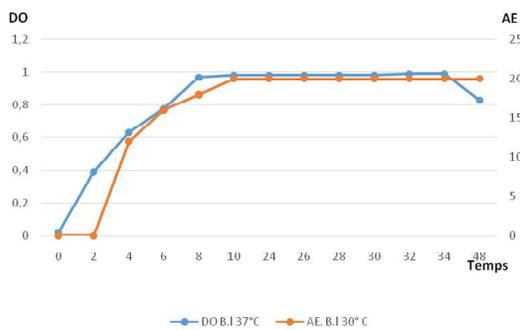


Fig. 8. Profiles of growth and enzyme production in *Bacillus licheniformis*

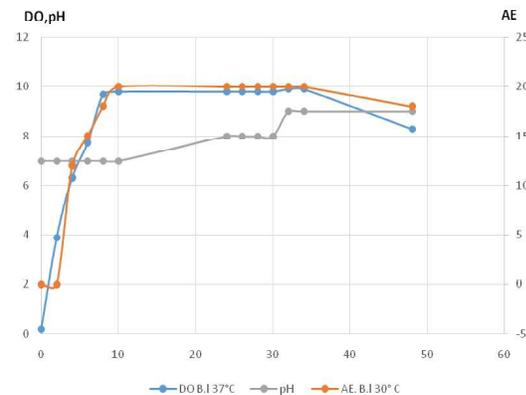


Fig. 9. Profiles of growth, enzyme production and pH variation during culture in the two studied strains

For *B. licheniformis*, the profile comprise:
 From 2 to 10h, an exponentially phase of enzyme production ;
 From 10 to 26h, a stationary phase of enzyme production ;
 From 26 to 30h, a decreasing phase of enzyme production ;
 From 30 to 48h, a second stationary phase of enzyme production:.

For *B. megaterium*, the profile comprise:
 From 2 to 8 h, an exponentially phase of enzyme production ;
 From 8 to 26 h, a stationary phase of enzyme production;
 From 26 to 48h, a decreasing phase of enzyme production ;
 From 34 to 48 h, une phase de diminution de production d'enzyme.

Fig-8 shows how evolve the enzyme production comparing to the growth in *B.li*. It is shown that the enzyme production is maximal during the stationary phase of the growth. This parallelism is the same for *B.me*.

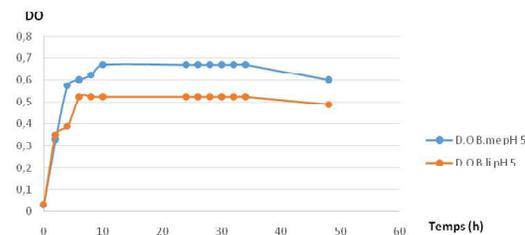


Fig. 10. Growth profiles of *Bacillus megaterium* and *B.licheniformis* at pH 5

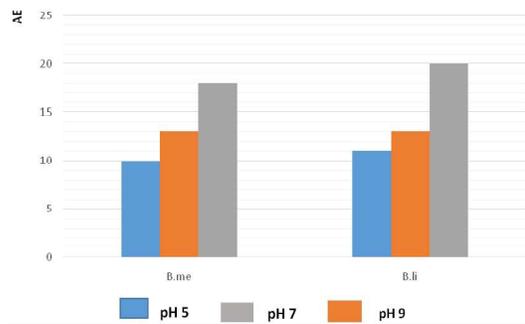


Fig. 12. Diagrams of caséinolytic enzyme production of *B.li* and *B.me* at different pH

Fig-9: shows that the pH increase rapidly at the end of the enzyme production phase, from 7 to 9 in the two studied strains.

Influence of pH of the medium in the growth and the enzyme production

Fig-10 shows the influence of pH of the medium on growth in the two strains. It is shown that growth is possible at acidic pH. The phases are the same in the two strains.

Fig-11 shows the influence of pH on growth in *Bacillus megaterium* and *Bacillus licheniformis*. It is shown that growth is possible in basic pH at 9, and the profiles comprises the same phases.

Fig-12 Shpows how caseinolytic enzyme production is possible in different pH of the cultured media The optimum is at pH 7.

Influence of different kinds of media on growth and the enzyme production

Fig-13 shows for the two strains growth profiles with the same phases. Growth is possible

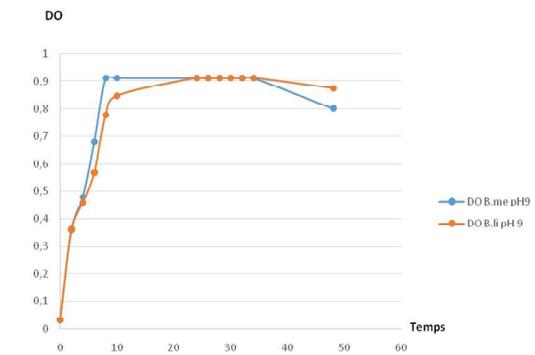


Fig. 11. Growth profiles at pH 9 in *Bacillus megaterium* et *B.licheniformis*

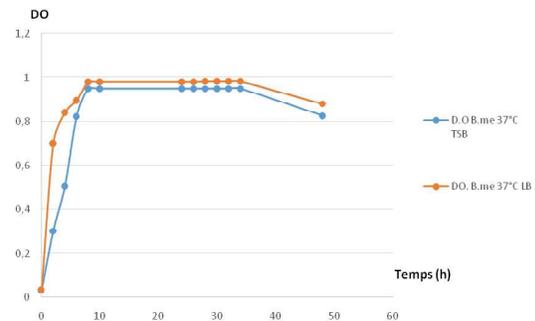


Fig. 13. Comparing growth profiles in different kinds of media for the two studied strains

for the two used media, the LB medium is better than TSB.

Fig-14: shows the same phases in the two caseinolytic enzyme production profiles.

For LB medium : a latency phase in short and the amplitude of enzyme production is important. For the TSB medium : a latency phase is important and the amplitude of enzyme production is less important than in LB.

Influence of different carbon sources on the growth and caseinolytic enzyme production

In [Table-3] are shown how growth and caseinolytic enzyme production are influenced by different carbon sources in the two studied strains. *Bacillus licheniformis* and *Bacillus megaterium*. Sugars are important for growth and enzyme production in the two strains. Fructose is used to be the best carbon source.

Influence of different nitrogen sources on the growth and the caseinolytic enzyme production

Among the two nitrogen sources used, yeast extracts is the best for growth in two strains (*B. licheniformis* and *B. megaterium*). For enzyme production both sources can be used in *Bacillus licheniformis*, in contrast only the yeast extract is efficient in *Bacillus megaterium*. The results are shown in Table 4

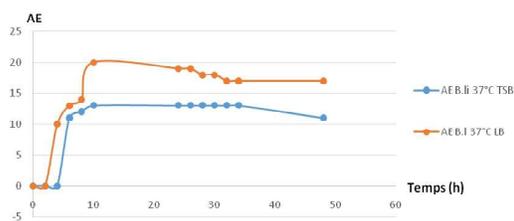
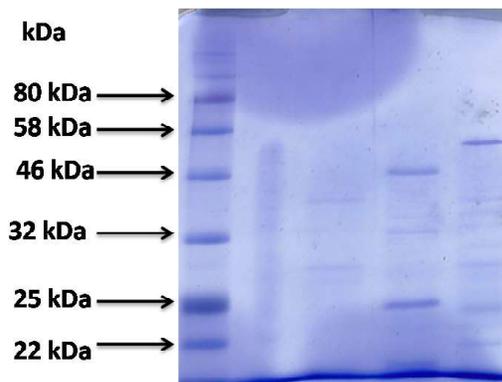


Fig. 14. Comparing profiles of caseinolytic enzyme production in two different media for the two studied strains

Figure 17 is showing the differentiation of the two bacillus strains by SDS-PAGE, this figure is showing clearly differences between the two strains.

DISCUSSION

The diameter of the halo testifying the caseinolytic enzyme activity is used in this work to express the enzyme production. The Temperature is an important factor for growth and the enzyme production. All growth profiles in the two strains (*B.licheniformis* and *B.megaterium*), with the temperature have the same phases, including exponentially phase, stationary phase



1: marker in KDa, 2:supernatant after culture of B.li, 3:supernatant after culture of B.Me, 4:ammonium sulfate precipitate of B.li supernatant after culture, 5: ammonium sulfate precipitate of B.me supernatant after culture. Lane 4, two important bands are displayed at 46kDa and 25kDa respectively. Lane 5 one important band is displayed on 56kDa.

Fig. 15. Proteins profiles on SDS-PAGE of two strains

Table 1. Different media with different carbon sources

Media	Carbon sources	Nitrogen source	Mineral solution
Medium 1	Galactose	Yeast extracts	MgSO4.5H2O: 0, 06%, KH2PO4 :0,1%, , 2 Cacl2.6H2O :0.2%, K2HPO4 .0, 2%
Medium 2	Fructose	Yeast extracts	MgSO4.5H2O: 0, 06%, KH2PO4 : 0,1%, Cacl2.6H2O:0.2%, K2HPO4 .0, 2%
Medium 3	starch	Yeast extracts	MgSO4.5H2O: 0, 06%, KH2PO4 : 0,1%, Cacl2.6H2O :0.2%, K2HPO4 .0, 2%

and the decreasing phase. **Figures 4 and 6.** All profiles related to caseinolytic enzyme production, have also the same phases, including the latency phase, an exponentially enzyme production phase, a stationary production phase, and the decreasing production phase, **Figures 5 and 7.** For *B.li*, the optimal temperature of growth is 37°C and the optimal temperature of enzyme production is 30°C. For *B.me* the optimal temperature of growth is between [35-37] °C, and the optimal temperature of enzyme production is 35°C. These results are similar to those already published [15, 12, 27].

The growth profiles have not shown a latency phase. The absence of latency phase is related to the used method. The OD measure is done just to hours after the inoculation of an overnight culture. These results are different to those published by [24] which presents a latency phase for different method of inoculation.

The growth and the enzyme production are different phenomena, this is shown on the figure 10. At all temperatures and for the two studied strains, the enzyme production is optimal during the stationary phase of the growth, these results are similar to those published in [12, 15, 27].

During the exponentially phase of growth, bacterial cells are dividing until the nutrients are still in the medium, when the nutrients go smaller and smaller in the amount, toxic products accumulate. This brings the changes in the culture conditions. **Figure 11** is illustrating how the pH of the medium has passed from 7 to 9. These results are identical to standard of bacterial culture and those published by [28].

For the two studied strains, the decreasing phase is followed by a stationary phase on which the OD value remained stable, and the caseinolytic enzyme production is still

Table 2. Different media with different nitrogen sources

Media	Nitrogen sources	Carbon source	Mineral solution
Medium 1	Yeast extracts	starch	MgSO ₄ .5H ₂ O: 0, 06%, KH ₂ PO ₄ : 0,1%, CaCl ₂ .6H ₂ O :0.2%, K ₂ HPO ₄ .0, 2%
Medium 2	Bactopeptone	starch	MgSO ₄ .5H ₂ O: 0, 06%, KH ₂ PO ₄ : 0,1%, CaCl ₂ .6H ₂ O : 0.2%, K ₂ HPO ₄ .0, 2%

[23, 25,26,].

Table 3. Effect of three carbon sources on the growth and the enzyme production

Carbon sources	Growth (DO at 600nm) 48h		Enzyme production (diameter of halo in mm) 48h	
	<i>B.li</i>	<i>B.me</i>	<i>B.li</i>	<i>B.me</i>
Galactose	0,903±0,002	0,849±0,001	13,33±1,24	11,33±0,47
Fructose	0,93±0,001	0,928±0,002	14,33±1,24	14,33±1,24
Amidon	0,711±0,001	0,845±0,004	10,00±0,81	16,66±1,24

Table 4. Effect of two different nitrogen sources on the growth and the caseinolytic enzyme production in *B.li* and *B.me*

Nitrogen sources	Growth (DO at 600nm) 48h		Enzyme production (diameter of halo in mm) 48h	
	<i>B.li</i>	<i>B.me</i>	<i>B.li</i>	<i>B.me</i>
Yeast extracts	0,969±0,015	0,952±0,01	14,333±1,247	14,333±0,471
Bactopeptone	0.514±0.003	0.739±0.002	14,333±1,247	11.666±0.942

important. This phase is assimilate to the sporulation, these results are in harmony with those related to *B. Licheniformis* and *B. megaterium*, which gradually go on sporulation, according to [29,30]

All the two strains can be cultured at different pH (5,7 et 9), with the optimum at 7. These results are different than those published by [31], which stipulate that these strains could not growth and can not produce the enzyme when the starting pH of the medium is at 5.

In the two used media, the LB is better rather for growth and fore enzyme production, on the two studied strains.

Among different carbon sources used. The best growth is observed with fructose and galactose which are monosaccharides. For polysacchrides growth and enzyme production are low.

When using the two sources of nitrogen, yeast extracts have been revealed to be better than bactopecton for growth in the two strains (*B.li* 0,969±0,015 *B.me* 0,952±0,01). In *B.li* the two nitrogen sources can be used for enzyme production (Yeast extracts: 14,333±1,247, bactopecton: 14,333±1,247), but in *B.me* only the yeast extract yield the important enzyme production. In deed in our laboratory, we have set the standard of the enzyme production, the reference strains *B.amy* yield on the fructose medium the standard growth of 0.954±0.001, and at 48h of culture the standard enzyme production is 13.333±0.001. It is according to these standard values, the strain is declared a caseinolytic producer (non published results).

We used SDS PAGE to differentiate the two strains through partial purification of protein in the supernatant after culture, and ammonium sulfate precipitation. The **Figure 16** is giving the protein profiles of the two studied strains. Specific bands can allow discrimination of the strains.

CONCLUSION

This article have helpful to our laboratory, for we have set new optimal conditions of two new strains in growth and caseinolytic enzyme production. We have discover the specificity of the two strains. Our methods has allowed to discriminate the caseinolytic producers by SDS-

PGE. This article has shown the importance of setting standard measure for enzymatic production. In a near future we will do the total purification and sequencing these caseinolytic enzymes.

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