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Production of Biosurfactants and Hydrolytic Enzymes by Bacillus-Genus Bacteria Isolated from Mbala Pinda, Traditional Food, Republic of Congo

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Abstract Mbala pinda is one of the traditional foods consumed in Congo. In this study, the objective of which was to analyze the production of biosurfactants and that of hydrolytic enzymes from five strains of the Bacillus genus isolated from Mbala pinda and identified by gene analysis and 16S rRNA. These five strains already present in GenBank are the following:IM1=*Bacillus safensis* MPRN8 (MT107116), IL1=*Bacillus megaterium* MPRN5 (MT107117), IN1=*Bacillus amyloliquefaciens* MPRN2 (MT107118), ID1=*Bacillus subtilis* MPRN7 (MT107119), IMa1=*Bacillus velezensis* MPRN1 (MT107120). These bacteria are cultured, the cultures are then centrifuged, the supernatant obtained is used to demonstrate the production of biosurfactants and hydrolytic enzymes. Finally, an evaluation of the production of biosurfactants and that of hydrolytic enzymes is made, the correlations for each strain are established between the production of biosurfactants and that of hydrolytic enzymes. The results obtained showed that all five strains produce revealable bioisurfactants from the supernatant of the cultures and both in the presence of gasoline and diesel. The emulsification indexes for the five strains tested are greater than 60% for gasoline and greater than 50% for diesel. The five strains produce proteolytic enzymes with higher productions for IN1 and ID1. The Five strains produce cellulolytic enzymes, IN1 and ID1 are the best producers in our working conditions. The five strains also produce amylolytic enzymes in significant proportions in comparison with the control strain, but here it is IMa1 the main producer and also IM1 and IN1 which also produce significant quantities of these enzymes. The PCA analytic correlations for the production of hydrolytic enzymes according to the different strains and their growth show that each strain has its own characteristics, but all produce these enzymes.

Keywords: *mbala pinda, Biosurfactants, hydrolytic enzymes, traditional food*

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1. Introduction

In the Congo, people eat both imported and local foods. In all the departments of the Congo, there are traditional foods that have retained their reputation and continue to be consumed by the populations. These traditional foods, far from being completely modernized, have remained at the level of local manufacturing units. However Congolese researchers try to make the scientific revaluation of certain foods by a certain number of studies, in particular [1], which worked on the microbiological composition and the aspects of fermentation on the following traditional fermented foods: Ntoba mbodi (fermented cassava leaves), potopoto (maize porridge), chikwangue cassava retted, wrapped and boiled). [2] have, through a review of the literature, made a non-exhaustive assessment of traditional fermented or non-fermented foods that can be revalorized in Congo and capable of playing an important role in the context of food security. Some of these foods such as crushed and fermented pepper was the subject of a study conducted by [3], specifying its microbiological composition during the fermentation process and also the dominant strains.

[4] also determined by pyrosequencing the bacteria responsible for fermentation and the dominant strains over time in Ntoba Mbodi.

In most of these traditional fermented foods, studies have shown that isolated bacteria, especially of the Bacillus genus, produce hydrolases, including [5], showing the production of fibrinolytic enzymes produced by strains isolated from Ntoba Mbodi, [6], showing the production of hydrolases by bacteria also isolated from Ntoba mbodi.

Proteases are one of the most important groups of industrial enzymes representing around 60% of the total industrial market. They are applicable in a wide range of commercial uses, such as detergent, leather, food and pharmaceutical industries [7].

Amylases are extracellular endoenzymes. This class of industrial enzymes accounts for approximately 25% of the enzyme market. These amylases are used in many applications in the textile, paper, brewing, baking, distilling, preparation of digestive aids, cake production, and pharmaceutical industries [8,9]

[10] showed that the production of cellulases was the most expensive step during the production of ethanol from cellulosic biomass, in the sense that it represented approximately 40% of the total cost. The chance of getting cheap ethanol will depend on the successful selection of a new cellulase-producing strain.

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants widely distributed in the environment. Microorganisms growing on aliphatic hydrocarbons secrete biosurfactants which facilitate the utilization of hydrophobic substrates. However, the isolation of bacteria producing biosurfactants during growth on PAHs represents a new aspect allowing a better understanding of the microbial mechanisms responsible for the degradation of PAHs. [11]

Classified as a traditional fermented food [2] Kayath et al., 2016, Mbala pinda is considered to be even less studied, it is a food made from the mixture of retted cassava paste, peanut paste and salt, sometimes hot pepper is added to it, the well-kneaded mixture is wrapped in the leaves and heated for cooking. It can be consumed immediately afterwards, but also stored in the sun. In Congo, this food is widely consumed in the departments of Bouenza and Niari, and Kouilou. Its percentage composition in nutrients is not yet specified in the literature. However, due to the presence of peanut paste, chilli and cassava whose compositions are known, it can be estimated that Mbala pinda is a food that provides a certain number of nutrients in the body.

The objective of this work was to analyze the production of biosurfactants and that of hydrolytic enzymes from five strains of Bacillus isolated from Mbala pinda and identified by 16S rRNA gene analysis . These five strains already present in GenBank are the following:IM1=*Bacillus safensis* MPRN8 (MT107116), IL1=*Bacillus megaterium* MPRN5 (MT107117), IN1=*Bacillus amyloliquefaciens* MPRN2 (MT107118), ID1=*Bacillus subtilis* MPRN7 (MT107119), IMa1=*Bacillus velezensis* MPRN1 (MT107120) In this work, Bacillus strains are cultured, the cultures are then centrifuged, the supernatant obtained is used to demonstrate the production of biosurfactants and hydrolytic enzymes. Finally, an evaluation of the production of biosurfactants and that of hydrolytic enzymes is made, the correlations for each strain are established between the production of biosurfactants and that of hydrolytic enzymes.

2. Materials and Methods

2.1. Bacterial Strains

The bacterial strains used in this study were isolated from samples of Mbala pinda isolated in a few localities in the Republic of Congo. These strains are in GenBank, they are listed below with their accession numbers:

Bacillus safensis MPRN8 (MT107116), *Bacillus megaterium* MPRN5 (MT107117), *Bacillus amyloliquefaciens* MPRN2 (MT107118), *Bacillus subtilis* MPRN7 (MT107119), *Bacillus velezensis* MPRN1 (MT107120).

2.2. Production Test of Biosurfactants by the Identified Strains

2.2.1. Emulsification Test (E24)

[12] developed the biosurfactant emulsification test (E24), which consists of adding a volume of Hydrocarbons (kerosene, gasoline, diesel, petroleum, etc.) to a volume of the aqueous sample. The mixture is homogenized using a vortex at high speed for 2 minutes. After 24 hours, the height (h) of the stable emulsion layer is measured. The calculated emulsion index E24 is the ratio of the height of the emulsion layer and the total height of the liquid

$$E_{24} = \frac{h_{emulsion}}{h_{total}} \times 100\%$$

2.2.2. Preparation of Lysogeny Broth Medium

1g of LB (lysogeny Broth) were weighed and mixed with 500ml of distilled water, after homogenization, the mixture was sterilized in an autoclave at 121℃ for 15 minutes. After cooling the medium, the strains to be tested were inoculated into five LB flasks (including one flask per strain, we have five isolated and identified strains) then incubated at 37℃ in a rotary oven (100 to 200rpm) overnight.

2.2.3. Protocol

Depending on whether the biosurfactant is bound to the cell membrane or directly excreted into the extracellular medium during growth.

In one of the cases, using overnight cultures, the strains were inoculated into five new LB media for 24 hours with shaking at 37°. The optical density of each culture is measured. 2ml of each culture are taken and mixed with 2ml of gasoline (or diesel), the mixture is vortexed for five minutes and finally left to stand for 24 hours at room temperature.

In the other case, after inoculating the cultures overnight at 37° in new LB with shaking for 24 hours, each culture is centrifuged at 600rpm for 10 minutes, after

which, 2ml of each supernatant is mixed with 2ml of gasoline in tubes sterile. Each mixture is homogenized and vortexed for five minutes and left to stand at room temperature for 24 hours. In either case, the total height of the oil (HT) and the height of the emulsion are measured in centimeters from a graduated ruler. The emulsion index is then calculated.

2.3. Hydrolytic Enzymes Production Tests

2.3.1. Culture Conditions and Optical Density Measurement

The production of hydrolases is done by growing the isolates and measuring the optical density which expresses the growth. For this purpose, a colony of each isolate is cultured in the LB medium with shaking, using a rotary incubator with 60 rotations per minute at 37℃. for 48 hours. Three (3ml) of the bacterial suspension are taken under sterile conditions, of which 2 ml were used to measure the optical density of the cell culture (growth) at 600 nm from a zuzi type spectrophotometer (Model 4211/50) [13]. 1ml is kept to be used for the detection and evaluation of the production of Hydrolases [14,15].

2.3.2. Production of Proteolytic Enzymes

To test the production of the enzyme with a proteolytic effect, casein or even skimmed milk are used (we speak of the caseinolytic effect). Modified techniques were used, according to [5,15].

One ml of the culture solution previously cultured at 37℃ for 48 hours was used to demonstrate and evaluate proteolytic enzyme production. In short, the casein box was used (skimmed milk = fat-free milk and therefore the main protein is casein). In a 250 ml Erlenmeyer flask containing 100 ml of PBS at 0.1N, dissolve 1 g of agarose, heat until completely dissolved, allow to cool to 55-60℃, add 10 ml of skimmed milk or 10 ml of casein to (0.2g/l)0, homogenize the mixture. Pour into Petri dishes; after solidification, prepare wells in the gel. Place 50µl of supernatant from the centrifugation of 1ml of culture in each well. Place the dishes in the oven at 37℃ for 12 hours. The observation of a clear translucent zone on the box containing the skimmed milk indicates that the strain produces a proteolytic enzyme with a caseinolytic effect (caseinolytic protease) [15,16,17]. The measurement of the diameter of each clear zone makes it possible to evaluate the production of enzyme.

2.3.3. Production of Cellulolytic Enzyme

Methyl cellulose carboxyl (CMC) was used, the composition for 100ml of distilled water is as follows 1g CMC, 0.2g Nacl, 0.1g NaNO3, 0.1K2HPO4, 0.1Kcl, 0.05g MgSO4, 0.5 g of yeast extract 1.7 g of Agar, the mixture was sterilized in the autoclave for 15 min at 1210C. During cooling to room temperature, the mixture was poured into the Petri dishes, the wells were formed in the gel after solidification.

50 µL of the supernatant were deposited in the wells. The dishes were incubated in an oven at 37℃ for 48 h. After which the dish was covered or soaked with lugol solution on the surface. After one minute, a translucent spot or clear halo was observed around the well indicating that the isolate possesses cellulolytic activity [18,19]. The measurement of the diameter of the clear zone around the well is used to assess the production of cellulolytic enzyme.

2.3.4. Production of Amylolytic Enzymes

Starch agar was used, briefly: 1 g of starch and 1.5 g of agar were dissolved in a vial containing 100 mL of distilled water. The mixture was sterilized in an autoclave for 15 min at 121℃. While cooling to room temperature, the gel was poured into petri dishes. The wells were formed on the gel after solidification [20,21].

50 µL of the supernatant were deposited in the wells. The dishes were incubated in an oven at 37℃ for 48 h. The dish was coated or soaked with lugol solution on the surface. After one minute, a translucent spot or clear halo was observed around the well indicating that the isolate possesses amylolytic activity [18,19]. The diameter of the clear zone makes it possible to evaluate the production of amylolytic enzyme.

3. Results

3.1. Capacity of the Strains Used to Produce Biosurfactants

3.1.1. Demonstration of the Production of Biosurfactants.

Figure 1 shows respectively in E, F and G the three strains IM1, IMa1 and IN1 whose supernatants in the presence of gasoline show emulsification illustrating the production of biosurfactants

All the three strain are showing the emulsification.

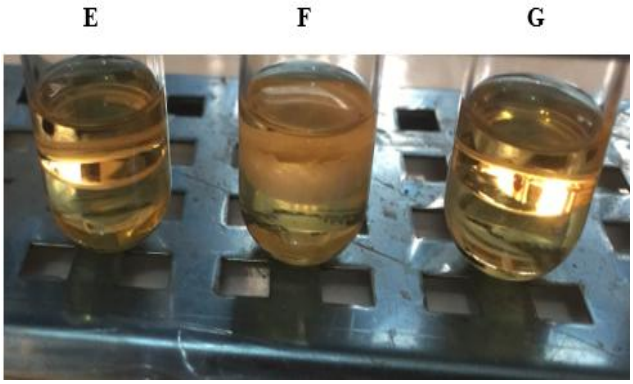

Figure 1. Illustration of Biosurfactants production by three strains if this study with gasoline: E for IM1, F for IMa1 and G for IN1

3.1.2. Evaluation of Emulsification Indexes in the Presence of Gasoline

Figure 2 shows the variation in emulsification indexes depending on the strains used in the presence of gasoline. In the presence of gasoline all the strains are able to emulsify, but at different percentages, so the indexes are different according to the strains with the IMa1 strain presenting the greatest index. Here emulsification occurred from the supernatant for all strains. The emulsification indexes are all above 60%, testifying to a significant power of production of biosurfactants for all the strains identified in this study.

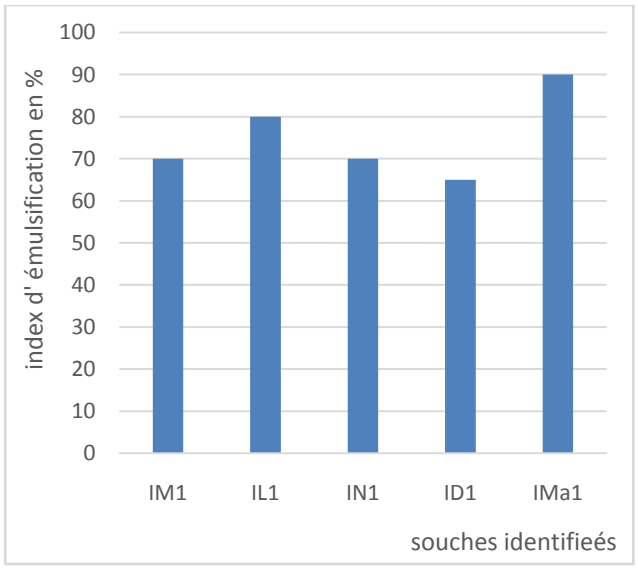

Figure 2. Profiles of emulsification index with gasoline of used strains in this study

3.1.3. Evaluation of Emulsification Indexes in the Presence of Diesel

Figure 3 shows the variation in emulsification indexes depending on the strains identified and in the presence of diesel. In the presence of diesel all the strains are able to emulsify, but at different percentages, so the indexes are different depending on the strains with the IMa1 strain having the highest index. Here emulsification occurred from the supernatant for all strains. The emulsification indexes are all above 50%, thus testifying to a great power of production of biosurfactants for all the strains identified in this study.

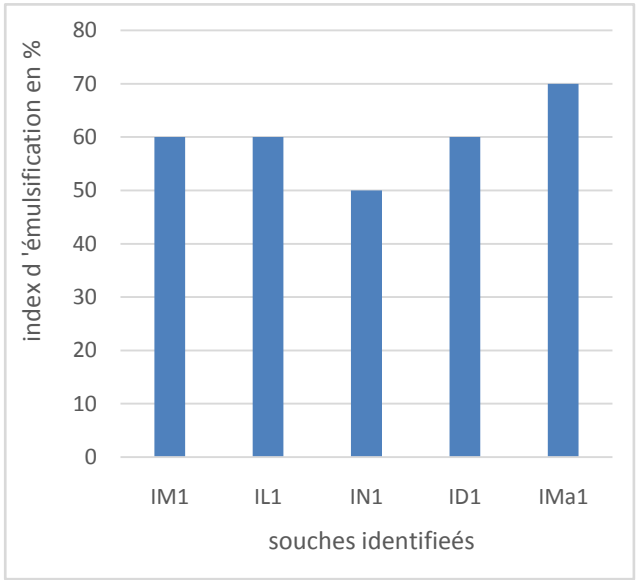

Figure 3. Profiles of emulsification index with diesel of used strains in this study

3.2. Production of Proteolytic Enzymes

3.2.1. Proteolytic Enzyme Production

Figure 4 shows the demonstration of proteolytic (caseinolytic) enzyme production by the identified strains. Apart from the negative control represented by E. coli, all the five strains of Bacillus identified produce the proteolytic enzyme. These are the different halos shown in the figure. Halos have different diameters.

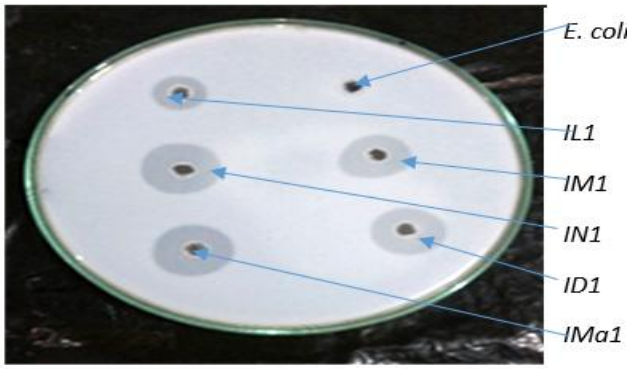

Figure 4. Halos illustrating the casein digestion by supernatants of différent cultures of strains used in this study.

3.2.2. Evaluation of Proteolytic Enzyme Production

Figure 5 shows the different Optical Density (OD) and cellulolytic enzyme production profiles of the identified strains. Under the culture conditions used, each strain identified shows growth. IL1 strains; IM1 and IMa1, however, show greater growth than that of the IN1 and ID1 strains. The IM1, IN1, ID1 and IMa1 strains produce quantitatively more enzyme than the IL1 strain.

Negative control *E. coli* shows interesting growth, but does not show any enzyme production under the conditions of our experiment.

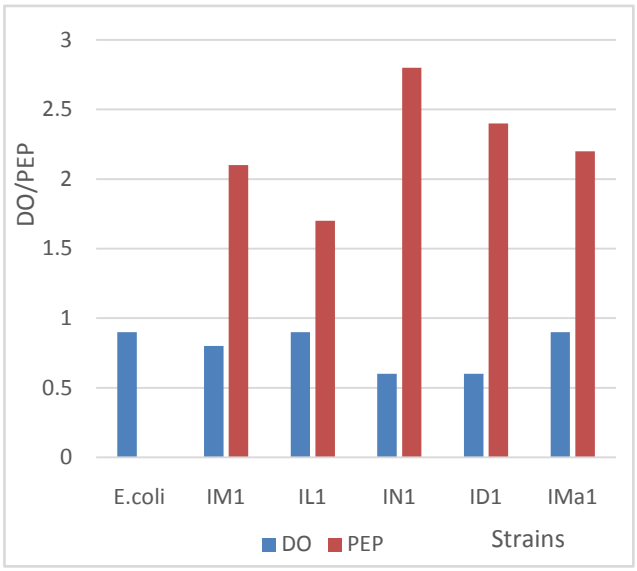

Figure 5. Profiles of growth (OD) and Proteolytic enzyme production (PEP) of used strains in this study

3.3. Production of Cellulolytic Enzymes

3.3.1. Cellulolytic Enzyme Production by the Identified Strains

Figure 6 shows the production of cellulolytic enzyme by the identified strains, it should be noted that all the strains produce the cellulolytic enzyme but in different quantities. The halos are clearly visible.

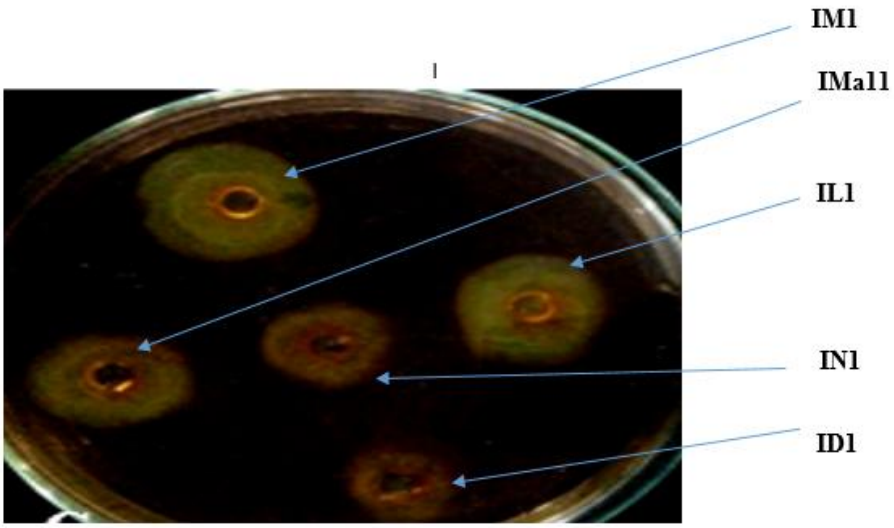

Figure 6. Halos of cellulose degradation by studied strains: IM1=Bacillus safensis MPRN8 (B.saf.), IMa1=Bacillus velenzis(B.v.) MPRN1, IL1=Bacillus megaterium (B.m) MPRN5, IN1=Bacillus amyloliquefaciens B.amy MPRN2., ID1=Bacillus subtilis(B.s.) MPRN7

3.3.2. Evaluation of Cellulolytic Enzyme Production

Evaluation of cellulolytic enzyme production of the identified strains. Under the culture conditions used, each strain identified shows growth. IL1 strains; IM1 and IMa1, however, show greater growth than that of the IN1 and ID1 strains. The IM1, IMa1 and IL1 strains produce quantitatively more enzyme than the IN1 and IDI strains.

E; coli was the negative control.

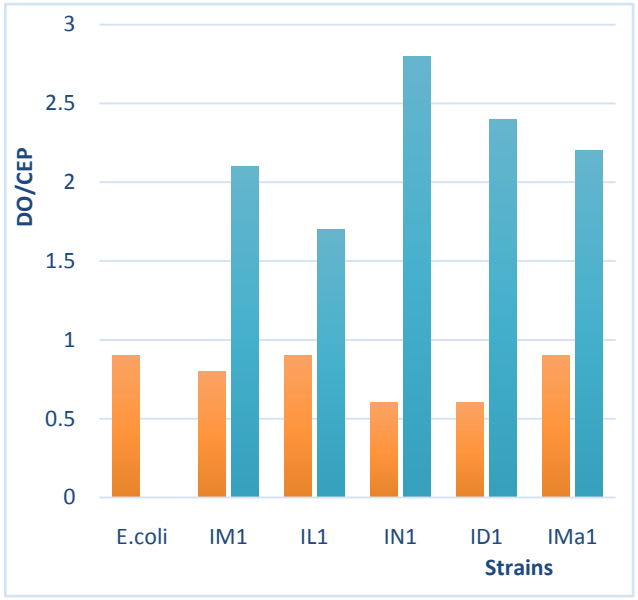

Figure 7. Profiles of growth (OD) and Cellulolytic Enzyme Production (CEP) of used strains in this study. In green the OD, in bleu CEP

3.4. Production of Amylolytic Enzymes

3.4.1. Demonstration of the Production of Amylolytic Enzymes

Figure 8 presents halos showing the digestion of starch by the supernatants of the cultures of the identified strains. Each strain identified is capable of producing the amylolytic enzyme under the conditions of the experiment. The production of amylolytic enzyme by each strain occurs in quantitatively different proportions, as evidenced by the difference in the diameters of the halos. The B. amEM24 strain is a reference strain from our laboratory whose amylolytic enzyme production capacities have been tested, this strain is used here as a positive control.

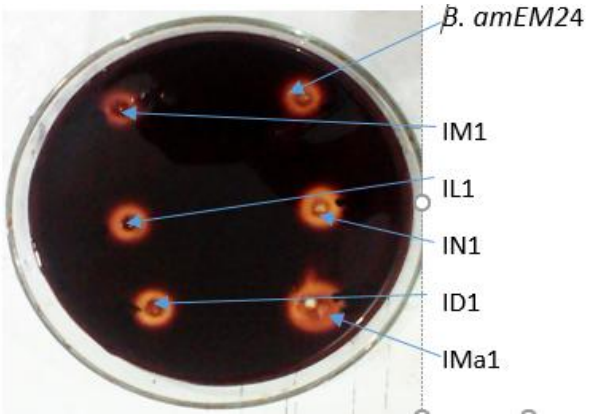

Figure 8. Halos of starch digestion by the studied strains

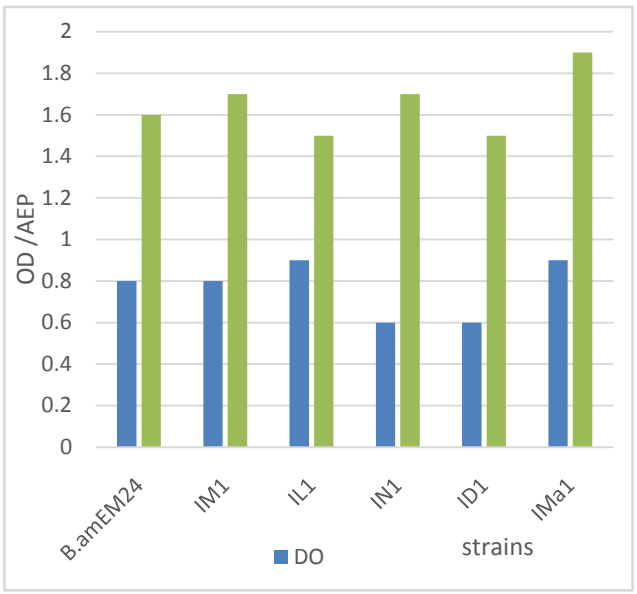

Figure 9. Profiles of growth and Amylolytic Enzyme Production (AEP) of strains used in this study. In grey = AEP

3.4.2. Evaluation of the Production of Amylolytic Enzymes

Figure 9 shows the variation in OD (Optical Density) and amylolytic enzyme production profiles of the different strains identified in this study. All the strains grow under the experimental conditions used and also produce amylolytic enzymes.

Taking into account the reference strain used as a positive control in this study, one can quantitatively say that: the IM1, IN1 and IMa1 strains produce more amylolytic enzymes than the reference strain, while the IL1 and ID1 strains produce less of amylolytic enzymes than the reference strain B. amEM24 (positive control strain).

3.5. Synthesis and Correlations between the Production of the Three Types of Enzymes for the Identified Strains

3.5.1. Principal Component Analysis (PCA)

The correlations for each strain concerning the growth and the production of proteolytic, cellulolytic and amylolytic enzyme were studied, Figure 10 shows the principal component analysis (PCA). The Figure shows that these correlations are well illustrated between the two PCA axes. Axis 1 contributes 64.5% in the analysis, while Axis 2 contributes 29.3%. IMa strains with its high production of the amylolytic enzyme. The IN1 strain shows a significant production of proteolytic enzyme.

This principal component analysis also shows that all the strains elaborate the three enzymes.

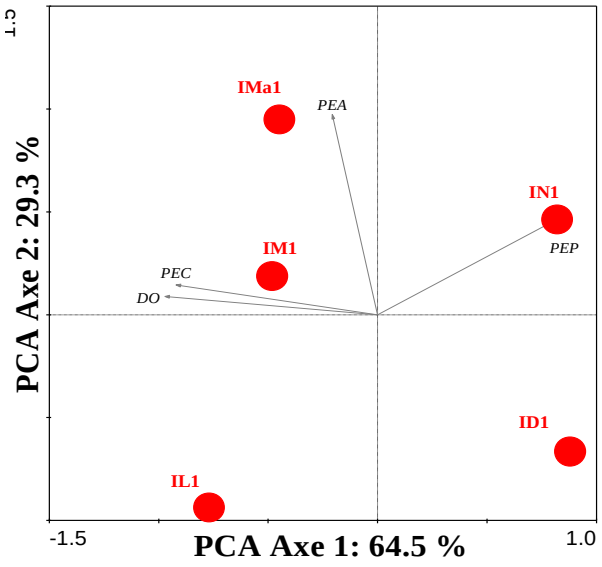

Figure 10. Principal Component Analysis (PCA) showing correlations between strains for OD and Hydrolytic Enzyme Production.

3.5.2. Identified Strains and Euclidean Distances for Enzymes Production

The Euclidean distance here makes it possible to see in terms of enzymatic production which strains are closer to the others and to confirm the results expressed in the various histograms and also to confirm the principal component analysis. Figure 11 clearly shows the distance between strains as a function of OD, and therefore of growth. Two distinct groups are observable, one formed by the IM1, IMa1 and IL1 strains and the other includes the IN1 and ID1 strains.

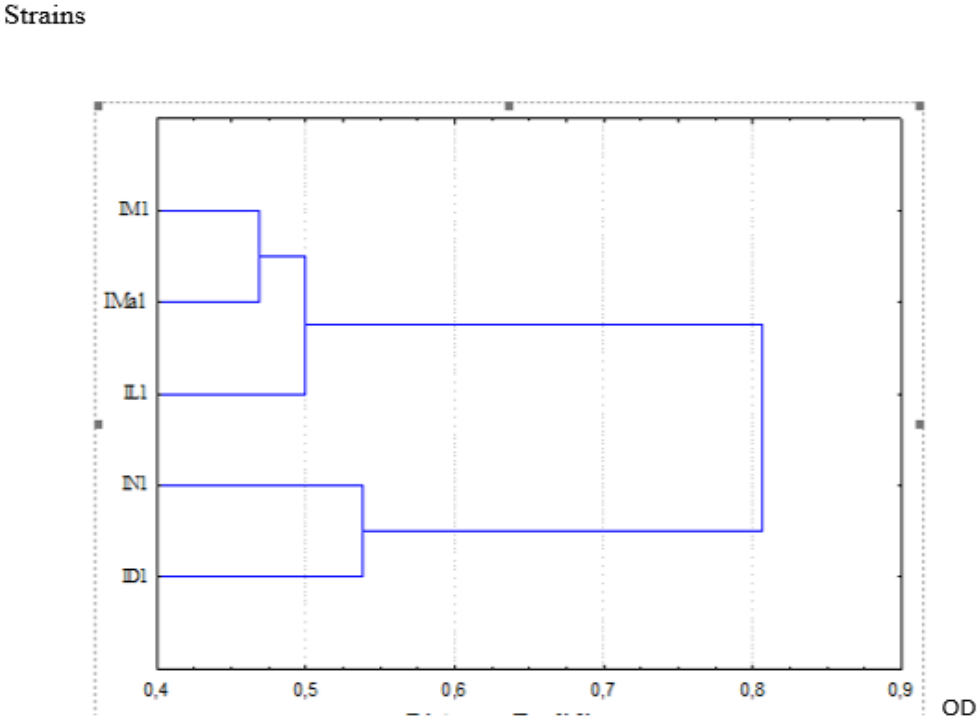

Figure 11. Euclidean distances between identified strains

4. Discussion

The objective of this study was to analyze the production of biosurfactants and that of hydrolytic enzymes (proteolytic, amylolytic and cellulolytic) by five strains isolated from Mbala pinda. These strains identified by the analysis of their 16S rDNA are deposit in GenBank and all belong to the genus Bacillus, the accession numbers are as following:

Bacillus safensis MPRN8 (MT107116), Bacillus megaterium MPRN5 (MT107117), *Bacillus amyloliquefaciens* MPRN2 (MT107118), *Bacillus subtilis* MPRN7 (MT107119), *Bacillus velezensis* MPRN1 (MT107120)

In this work, the strains used are capable of producing hydrolytic enzymes, in particular proteolytic enzymes, amylolytic enzymes and also cellulolytic enzymes. These results are close to those of [22], who also isolated bacteria in fermented foods that also produced these three types of enzymes simultaneously. Indeed [22], uses an approach, where the bacteria are placed in the presence of the substrate for the production of the different hydrolytic enzymes. This seems normal, because bacteria are known in such a way that they develop in the presence of a given substrate the enzymes capable of hydrolyzing this substrate. These results were also obtained by [6,23], again bacteria produce hydrolytic enzymes in the presence of the proposed substrate. However, if Starch induces amylase production, there are reports that starch may not be required for amylase production, probably in organisms with constitutive enzymes [24]. In reality in this work, using LB, this assertion would be understandable, because the bacteria identified here produce proteolytic enzymes, because they are in the presence of a medium containing casein, a protein inducing the production of proteolytic enzymes. On the other hand, what can be said for these bacteria which, placed in this same medium, also produce cellulolytic and amylolytic enzymes in the absence of these substrates? this is where the bacteria used here are assumed to have constitutive enzymes. Indeed, these bacteria identified in this work cultured in a medium where proteins predominate, showed in their supernatants the presence of cellulolytic enzymes and amylolytic enzymes. These enzymes (cellulolytic and amylolytic) are therefore produced in the absence of the substrate, thus illustrating the concept of constitutive enzymes. Indeed, in the absence of the substrates concerned, these bacteria were able to develop the related enzymes. The presence of the genes encoding these enzymes (cellulolytic and amylolytic) are certainly directly transcribed and translated during growth, it is here bacterial growth which is one of the factors controlling the expression of these genes (genes encoding the cellulolytic and amylolytic enzymes).

Finally, it is also necessary to take into account the culture conditions used which allowed the production of the hydrolytic enzymes. No optimization of the enzyme production conditions was made in this work, however many authors have shown that the culture conditions strongly influence the enzyme production in bacteria, this is the cat of [6,14,15]. In this work, the bacteria used produce cellulolytic enzymes, these results are close to those found by [25] who work on the soils under the same culture conditions also found cellulolytic bacteria. [4], also working on Ntoba Mbodi under the same culture conditions also found producing-hydrolases. bacteria [26] stated that: Biosurfactants are produced by a wide variety of microorganisms, including bacteria, yeasts and fungi, using different substrates such as carbohydrates, hydrocarbons, vegetable oils and glycerol for cell growth. In this study strains producing biosurfactants were used, they belong to the genus Bacillus, identified by the analysis of the 16S rRNA gene. The emulsification indexes are above 50%. These results confirm the rule of the production of biosurfactants by bacteria. These results are consistent with those of [27] who isolated a halothermotolerant Gram-positive spore-forming bacterium from oil reservoirs in Iran and identified as Bacillus licheniformis sp. ACO1 strain by phenotypic characterization and 16S rRNA analysis. This strain also showed a high capacity for the production of biosurfactants with an emulsification index of 90%. Indeed, the production of biosurfactants naturally by microorganisms has significant advantages in their applications. From the point of view of the production of biosurfactants by bacteria, [27,28] showed that due to various functional properties such as emulsification, wetting, foaming, cleaning, phase separation, surface activity and hydrocarbon reduction viscosity, biosurfactants are better used than synthetic surfactants in bioremediation, enhanced oil recovery, cleaning of pipes and vessels. In reality biosurfactants are environmentally friendly, biocompatible, less toxic with bio-degradability and specifically actives.

5. Conclusion

This study shows that bacteria isolated from Mbala pinda, a traditional food, are capable of producing not only biosurfactants, but also hydrolytic enzymes of different kinds, namely: proteolytic enzymes, cellulolytic enzymes and amylolytic enzymes. All these biomolecules are of biotechnological interest. This study opens up good prospects for the exploration of our traditional foods in connection with the purification of enzymes of interest that can help our country in the manufacture of detergents and many other useful products for our society.

Conflict of Interest

The authors declare no conflict of interest.

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