



SCREENING, ISOLATION AND PARTIAL STUDY OF CELLULASE PRODUCING MICROORGANISMS FROM SOIL SAMPLES FROM SAW MILLS OF THE CITY OF DOUALA CAMEROON.

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Keywords; Carboxymethyl cellulose, Cellulase; cellulosic biomass; sawmill, Douala Cameroon

ABSTRACT

During the last century, industrial development has been accompanied by a heavy dependence on fossil fuels, the use of which impacts the environment through the production of greenhouse gases. That is why public authorities are concentrating their efforts on finding renewable energies that are less polluting for the environment, such as the biotransformation of cellulose into second generation ethanol using an enzyme; cellulase (hydrolysis) and yeast (alcoholic fermentation). This work is part of the contribution to the establishment of a protocol permitting the production of cellulase at a lower cost for the purpose of an application in the production of second-generation bioethanol. Thus, this work was aimed to isolate a cellulase-producing microorganisms in two sawmills of Douala Cameroon, having over 10 years age. Then a screening was carried out in order to select among the isolates obtained the best isolate which has finally been partially characterized. Results showed that, out of 53 isolates obtained from the soil samples of the two sawmills, B12 isolate proved to be the best producer of cellulase with a CMC_{ase} activity of 0.026 ± 0.0016 IU/ml and an APFase activity of $0,0113 \pm 0.0014$ IU/ml. It would be a Gram- catalase positive shell bacterium and preferably develops at 45°C, pH 7, in a medium comprising 2% (m / v) of CMC; 2.5% (m / v) of casein and 0.6% (m/v) of potassium phosphate.

1. INTRODUCTION

Reducing the use of fossil fuels in the 21st century is one of the major challenge in the industrial developpement. As the demand for energy grows more and more, the use of fossil fuels is a source of atmospheric pollution like the emission of greenhouse gases [4]. In addition, the decrease in consumption of fuel would restrict the use of increasingly limited petroleum resources, and reduce the energy dependence of developing countries. It would therefore be interesting to turn to research into alternative energies like second-generation bioethanol resulting from the transformation of cellulosic biomass by a process of hydrolysis and fermentation in which cellulase is the main enzyme used. In fact, with the energy crisis of 1973, the public authorities took a keen interest on renewable resources. As such, cellulase was naturally at the center of research for the production of alternative fuels (methane, alcohols) [11]. Cellulose is a substrate available in nature and in inexhaustible quantities (plant), its valorization for the purpose of industrial production of bioethanol would be an interesting alternative because the use of second-generation bioethanol reduces greenhouse gases emissions. Moreover, its production does not interfere with human nutrition, as is the case with first generation bioethanol.

It is in view of all this that we are considering producing second-generation bioethanol from cellulose. To achieve this, we started by isolating cellulase-producing microorganisms from soil samples taken in two sawmills in the city of Douala then these microorganisms have been subjected to a screening in order to select the best producer who was partially characterized.

2. MATERIALS AND METHODS

2.1 Sampling

Soil samples were taken from two sawmills of Douala of over 10 years of age, located in Bépanda and Dibamba. The samples have been taken at the depositing site of wood chips 5 cm transported from the ground to avoid possible exogenous contamination [22] and carried in ice bath to the laboratory.

2.2 Enrichment

3g of soil from each soil sample had been introduced separately into sterile 100 mL flasks containing 30 mL of sterile salted carboxymethylcellulose (CMS) medium prepared according to the modified protocol of [17], containing: CMC 1.5% ; KH_2PO_4 0.2%; MgSO_4 0.02%; NaCl 0.02%). The pH of the medium was adjusted to 7 with NaOH for the isolation of bacteria. The whole was homogenized and incubated at 30°C in a shaker for 24 hours. The same conditions were performed for the isolation of the fungi except that the medium was supplemented with Chloramphenicol and the pH adjusted to 4 with HCl.

2.3 Isolation and purification

After 24 hours of incubation, the enrichment media was diluted successively (10^{-3} , 10^{-9} and 10^{-12}) then 100 μL of each dilution was spread in Petri dishes containing the CMC solid isolating media -agar (CMC 0.5%; KH_2PO_4 0.2%; MgSO_4 0.02%; NaCl 0.02%; agar 1.5%; $(\text{NH}_4)_2\text{SO}_4$ 0.14%) for bacteria and PDA-Chloramphenicol for fungi. The media were incubated for 24-48 hours at 30°C [7]. The purification of isolates obtained was carried out by successive subculturing of each differentiated colony on CMC-agar medium for the bacteria and PDA supplemented with chloramphenicol for the fungi. [12].

2.4 Selection of the best cellulolytic isolate

With the aim of choosing the most efficient cellulase-producing isolate, the isolates obtained were successively subjected to a series of two tests in liquid medium, including the test for determining the percentage reduction in the viscosity of the medium and the test for determining enzymatic activity after fermentation. The tests were carried out using the following protocol:

2.4.1 Preparation of the inoculum

The preparation of the inoculum was carried out by introducing colonies of isolates taken from the Petri dishes into CMSI medium (CMC 0.5%; KH_2PO_4 0.2%; MgSO_4 0.02%; NaCl 0.02% $(\text{NH}_4)_2\text{SO}_4$ 0.14%). The pH is brought back to 7 using soda and a Hanna brand pH meter. The whole was incubated at 30°C for 24 hours. After incubation, the inoculum obtained was calibrated at 1.5×10^8 CFU / mL by reading on a spectrophotometer and diluting so as to obtain an optical density of 0.10 at 625 nm [30].

2.4.2 Evaluation of the percentage reduction in the viscosity of the liquid medium after fermentation

For this test, 3 mL of inoculum from each isolate were introduced into 27 mL of CMS medium for a final volume of 30 mL [10]. The pH of the medium was adjusted to 5 for fungi and 7 for bacteria. The whole was incubated at 30 ° C in a shaker for 96 hours.

The viscosity of the medium had been determined using a viscometer in which we introduced 20 mL of each fermented medium and the flow time of the medium of each isolate was recorded in seconds. At the same time, the flow time of distilled water as well as that of a medium prepared under the same conditions as the fermentation medium but in which the inoculum is replaced by sterile distilled water was determined in order to serve as Indicator. The enzymatic activity, expressed in degree of viscosity, was obtained by applying the formula used by [3] which allowed us to preselect a few isolates.

$$V = \frac{T_0 - T}{T_0 - T_{eau}} \times 100$$

V = viscosity reduction percent,
T₀ = flow time of the unseeded medium
T = flow time of the culture filtrate
T_{eau} = water flow time.

2.4.3 Evaluation of the enzymatic activities of the preselected isolates

2.4.3.1 Production of crude enzyme

To produce crude enzyme, the fermentation media of the preselected isolates were prepared as described in section 2.4.2 and incubated at 30°C in a water bath for 96 hours. After fermentation, 10 ml of the fermented media of the preselected isolates were taken and placed in sterile test tubes and then subjected to centrifugation at 3500 rpm for 10 minutes. The recovered supernatant is considered as a crude enzyme for the determination of the enzymatic activities proper[25].

2.4.3.2 Carboxymethyl cellulase activity (CMCase) and filter paper activity (APFase)

The enzymatic activities had been determined according to the method described by [9]. The reaction mixture consists of 0.5 mL of enzymatic solution, added to 1 ml of phosphate buffer (0.05M) containing 1% of CMC for CMCase activity and 0.5 mL of enzymatic solution, added to 1 ml of phosphate buffer (0.05M) containing 1cm² of Wattman N° 1 paper for APFase activity. The enzymatic reaction was initiated by incubating the reaction mixture in a water bath at a temperature of 40°C for 10 minutes [6]. The reaction is stopped by adding the DNSA, then the reaction mixture is boiled for 5 minutes at 100°C, then cooled in an ice bath. Optical density was measured with a spectrophotometer at 540 nm. The quantity of reducing sugars released was determined using a calibration curve produced from a 0.1% glucose solution established with varying glucose concentrations from 0 to 0.01 g / L [19].

2.5 Partial identification of the best cellulase-producing isolate

The partial identification of the best isolate focused on the study of macroscopic characters, the microscopic study and some biochemical tests.

❖ **Macroscopic study:** it was based on the observation of colonies with the naked eye in a petri dish. Characteristics are observed: Colony borders, colony size, diameter, chromogenesis (color), elevation, opacity, area, colony appearance and consistency.

❖ **Microscopic study:** the microscope observation was carried out with objectives 40 then 100. This examination made it possible to verify the purity, the form (bacillus or Cocci) of the isolate, the mode of association, the mobility as well as their appearance. After Gram stain. This method makes it possible to differentiate gram + bacteria from gram- according to the protocol in Table 1 and the slides were observed at objectives 40 and 100 (addition of immersion oil) of the microscope [23].

❖ **Catalase test:** Catalase is an enzyme which intervenes in the breaking down hydrogen peroxide into oxygen and water. After spreading a colony on a clean glass slide; a drop of hydrogen peroxide is applied using a Pasteur pipette. A positive reading for the presence of catalase is translated within seconds by the apparition of air bubbles mainly due to the production of oxygen [28].

2.6 Influences of certain physicochemical parameters on cellulase production

In order to determine the optimum conditions for cellulase production, the best isolate was inoculated into 100 ml flasks containing 30 ml of fermentation medium and incubated in a shaker at 30°C for 96 hours. The influence of different growth parameters like: temperature, pH, CMC concentration, nitrogen sources, phosphorus sources and its concentration and the optimal fermentation time was studied. At the end of each fermentation, the cellulosic activity was evaluated according to the protocol described in paragraph 2.4.3.2.

❖ **Influence of the pH:** the determination of the optimum pH was performed by introducing 3 ml of inoculum ($1.5 \cdot 10^8$ CFU / mL) of the B12 isolate into 27 ml of the CMS medium at pH 2, 3, 4..... 12. The media were then incubated for 96 hours at 30°C.

❖ **Influence of temperature:** the determination of the optimum temperature was carried out under the same conditions as that of the pH, but this time the initial pH of each medium was maintained at 7 and then incubated for 96 hours at the following temperatures 25; 30 ; 35; 40, 45; 50°C and 60°C.

❖ **Influence of the nitrogen source:** in order to determine the best nitrogen source for optimal efficiency of the B12 isolate, 3 ml of inoculum ($1.5 \cdot 10^8$ CFU / ml) of the best isolate is introduced into 27 ml of CMS media containing as sole source of nitrogen respectively; yeast extract, peptone, urea, soy, casein and ammonium sulfate at a concentration of 0.3% (w/v). Each fermentation medium was incubated for 96 hours at 30 ° C.

❖ **Influence of the casein concentration:** the determination of the best casein concentration was carried out by introducing 3 ml of inoculum ($1.5 \cdot 10^8$ CFU / ml) in 27 ml of CMS medium containing casein as the only source of nitrogen at concentrations: 0.5%; 1%; 1.5%; 2%; 2.5% and 3%. The media were then incubated for 96 hours at 30 ° C.

❖ **Influence of the source of phosphorus:** the determination of the best source of phosphorus was carried out under the same conditions as that of nitrogen, replacing the sources of nitrogen with 0.06% (m/v) of disodium phosphate (Na_2HPO_4), of potassium phosphate (KH_2PO_4), and orthophosphoric acid (H_3PO_4).

❖ **Influence of the phosphorus concentration:** The determination of the best disodium phosphate concentration was carried out by introducing 3 ml of inoculum ($1.5 \cdot 10^8$ CFU / ml) of the best isolate in 27 ml of the CMS medium containing different concentrations of phosphorus: 0.0%; 0.2%; 0.4%; 0.6%; 0.8% and 1% . The media were then incubated for 96 hours at 30°C.

❖ **Influence of the CMC concentration:** the determination of the optimal CMC concentration was carried out by introducing 3 ml of inoculum ($1.5 \cdot 10^8$ CFU / ml) of the best isolate into 27 ml of CMS media prepared at concentrations: 0.5%; 1%; 1.5%; 2%; 2.5% and 3% (m / v) CMC. [8].

❖ **Influence of the fermentation time:** the optimum fermentation time was determined under the same conditions as that of the pH, but this time the initial pH of each medium was maintained at 7 and then incubated at 30°C for: 24 ; 48; 72; 96; 120; 144 and 168 hours. [18]

2.7 Statistical analysis

Data were introduced into an Excel sheet and then analyzed with the STATGRAPHICS Centurion XV software. One-way ordered analysis of variance (ANOVA) was used to compare mean values of enzyme activity across isolates. Non-parametric kruskal-whallis test was used to compare mean values of enzyme activity according to physicochemical parameters. Significance level was set at a probability value less than 0.05 (5%).

3. RESULTS

3.1 Isolation

Soil samples collected in the two sawmills allowed us to have 53 isolates of which 18 bacteria and 35 fungi (10 filamentous and 25 yeasts) as distributed in the table below.

Table 1: Distribution of isolates obtained according to sampling sites

Sampling sites	Number of samples	Number of isolates
Bépanda	4	Bactéria: 11 Mycetes: 16
Dibamba	4	Bactéria: 7 Mycetes: 19

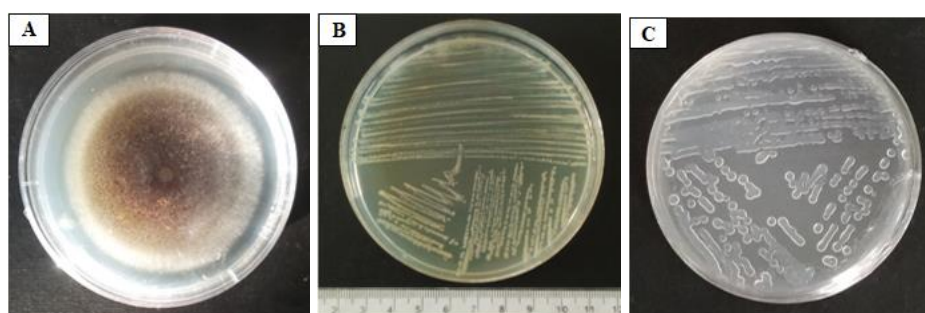


Figure 1. Presentation of some isolates: filamentous (A), bacterial (B) and yeast (C).

3.2 Screening for the best cellulase-producing isolate:

The two screening tests used here gave us the following results.

3.2.1 Test to reduce the viscosity of the liquid medium after fermentation:

Results of the viscometer test shown in Figures 2a and 2b give the enzymatic activity expressed as a percentage reduction in the viscosity of the fermentation medium of the isolates obtained. This test allowed us to preselect 16 isolates, those whose percentage reduction in the viscosity of the fermentation medium was greater than or equal to 90%. The choice of this test is linked to the fact that CMC is a gelling agent whose degradation in the medium is linked to a progressive decrease in viscosity resulting in a more or less liquid medium.

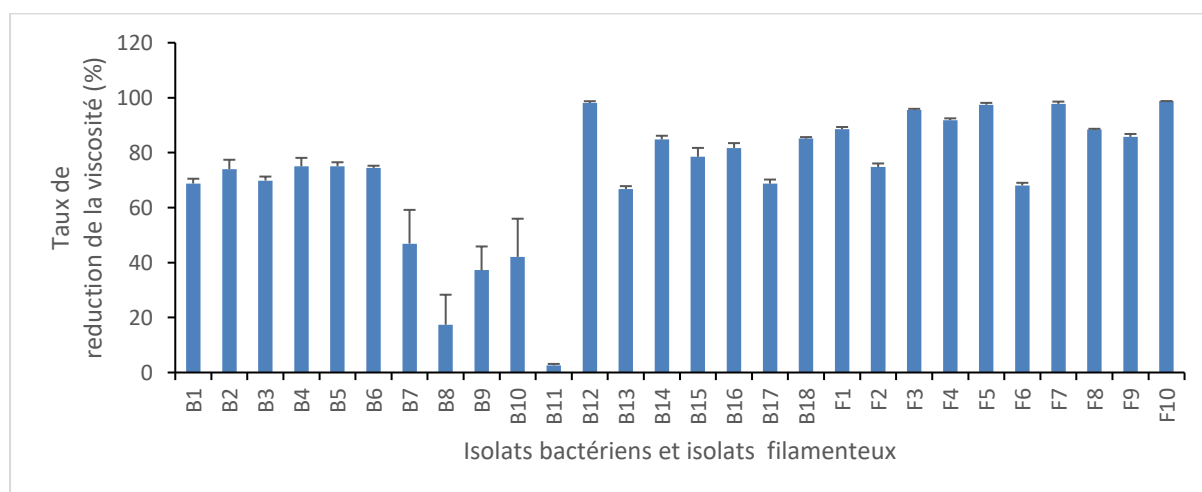


Figure 2a. Percentage decrease of the viscosity in the fermentation medium of bacterial and filamentous isolates.

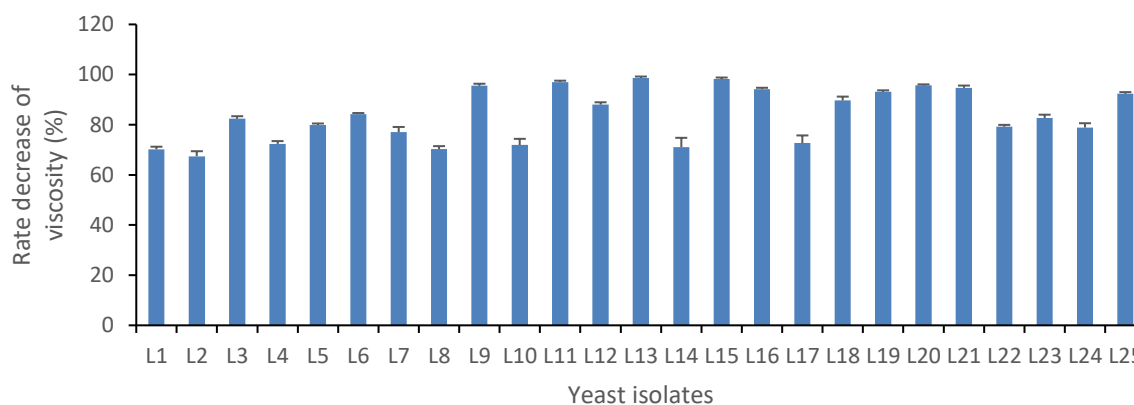


Figure 2b. Percent reduction in the viscosity of the fermentation medium of the yeast isolates

3.2.2. Evaluation of cellulase activities (CMCase and APFase)

APFase and CMCase-type enzymatic activities obtained for the 16 preselected isolates are presented in fig.3. It emerges from this that the tested isolates exhibit CMCase and APFase activities which vary respectively between 0.0017-0.0265 IU / mL and between 0.0013-0.0113 IU / mL. At the end of this test, the B12 isolate proved to be the best producer with a production of 0.0265 ± 0.0016 IU / mL for the CMCase activity; and 0.0113 ± 0.0014 IU / mL for APFase activity.

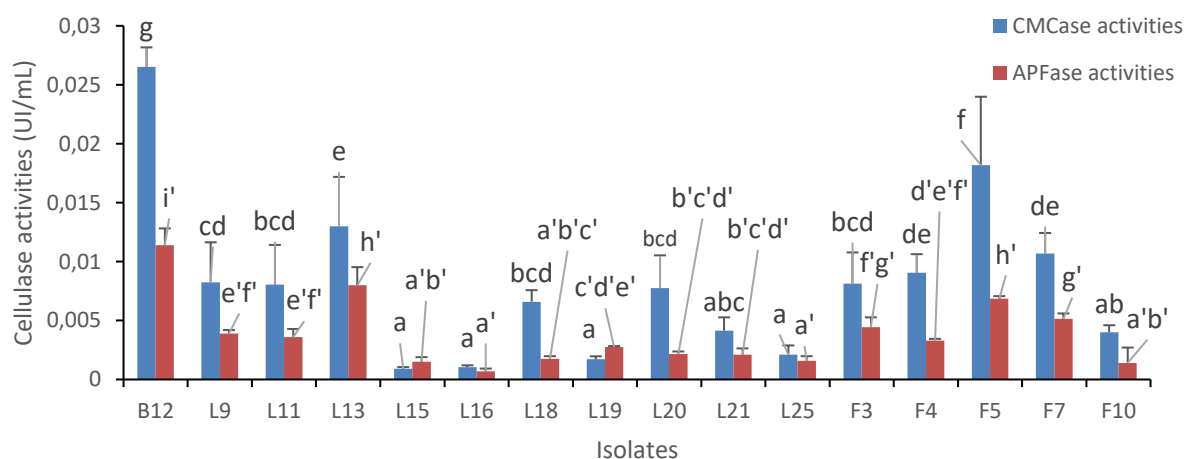


Figure 3. CMCase (■) and APFase (■) activities of the 16 isolates in liquid fermentation on CMC medium.

3.3 Partial identification of B12

Results of the macroscopic (A), microscopic (B) and catalase test (C) observations of the best isolate are shown in the figure below. Isolate B12 was found to form colonies with a rounded border with a creamy surface and a milky-yellow color. Under the objective 100 optical microscope (Gram stain), the latter appears as a pink shell and finally it forms a foam in the presence of hydrogen peroxide. In view of all this, this isolate would be a gram negative, catalase positive shell bacteria.

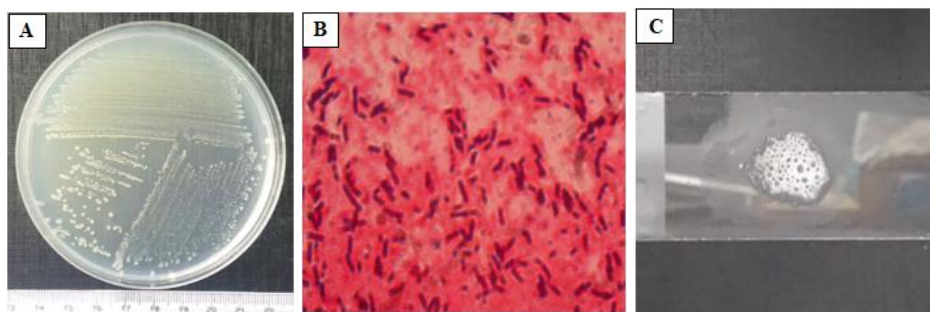


Figure 4. B12 isolate seen in a petri dish, under an optical microscope and subjected to the catalase test.

3.4 Influence of some physico-chemical parameters on cellulase production

3.4.1 Influence of the initial pH on the enzymatic production

The influence of the initial pH on the production of cellulase at 30°C is shown in Figure 5 below. This figure has a bell-like appearance and illustrates a gradual increase in activity ranging from pH 5 (0.0013 ± 0.0002 IU/mL) to pH 7 where the activity peaks (0.0167 ± 0.0009 IU/mL). Beyond this pH, there is a gradual decline in activity as pH increases. In addition, the differences observed are significant (F=110.13 and p < 5%)

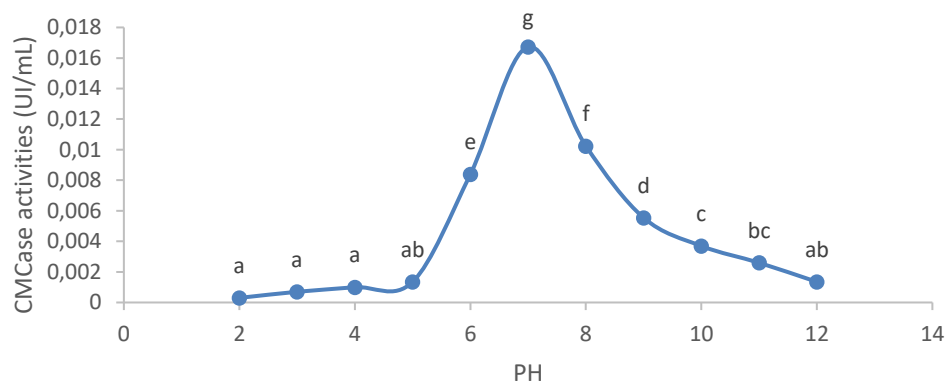


Figure 5. Influence of the initial pH on the enzyme production.

3.4.2 Influence of temperature on enzyme production

Figure 6 shows the influence of temperature on enzyme production by B12. It is noted that the amount of enzyme gradually decreases from 30°C to 45°C where it reaches its maximum (0.0224 ± 0.0014 IU/mL) and then gradually decreases as the temperature rises. Statistical analyses show that the differences in activity observed are significant (F=112.4 and p<5%)

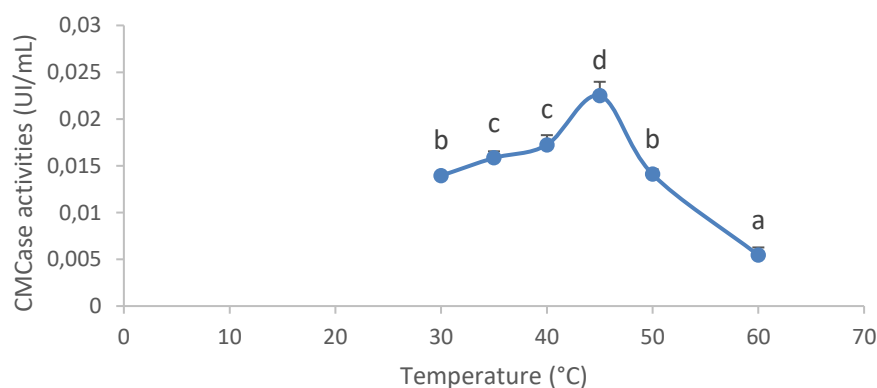


Figure 6. Influence of temperature on enzyme production.

3.4.3 Influence of the nitrogen source on the enzyme production

Figure 7 shows the influence of the nitrogen source on enzyme activity by isolate B12. The result is that casein has the best enzyme activity (from 0.0113 ± 0.0018 IU/mL) compared to other nitrogen sources. Also the differences observed are statistically different ($F = 17.40$ and $p < 5\%$)

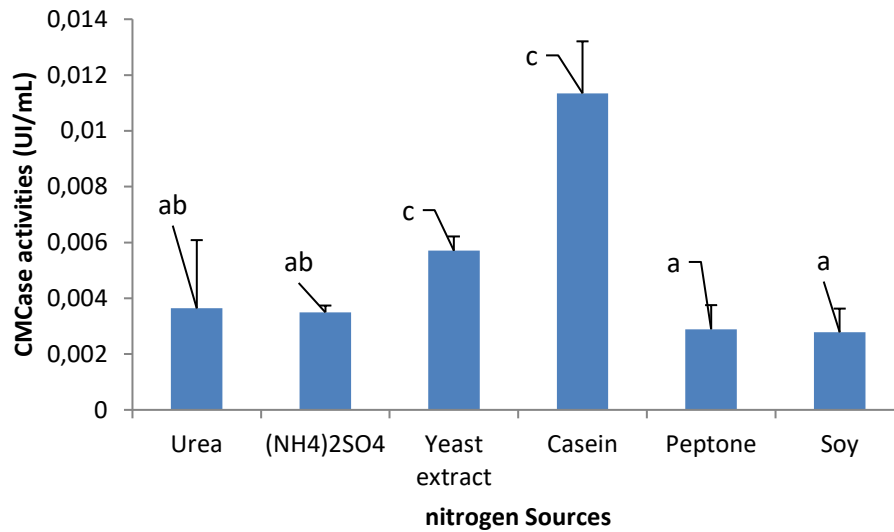


Figure 7. Influence of the nitrogen source on the enzyme production.

3.4.4 Influence of the casein concentration on enzyme production

Figure 8 shows the influence of casein concentration on the production of the enzyme by B12. The activity of the crude enzyme produced increases rapidly until reaching the maximum value (0.0298 ± 0.0001 IU / mL) at a concentration of 2.5% casein. Beyond these concentrations, activity gradually decreases.

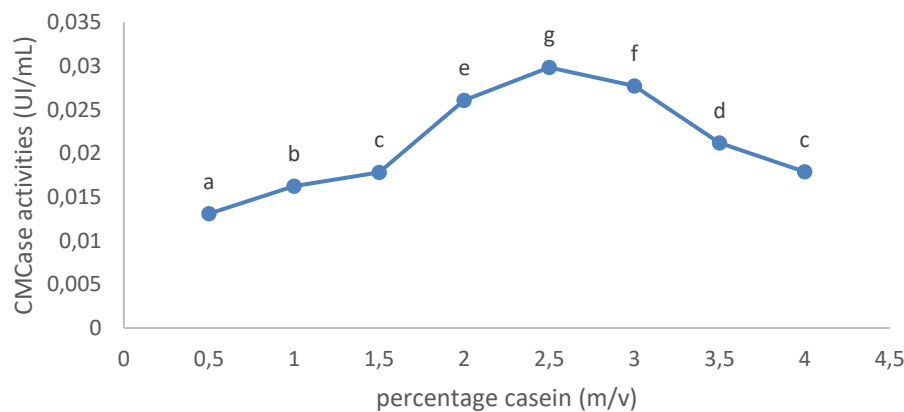


Figure 8. Influence of casein concentration on enzyme production.

3.4.5 Influence of the source of phosphorus on enzyme production

Figure 9 shows the influence of the phosphorus source on enzyme production by the B12 isolate. KH2PO4 has better cellulase production (0.0175 ± 0.0002 IU/mL) compared to other sources of phosphorus with significant differences ($F = 1387.75$ and $p < 5\%$).

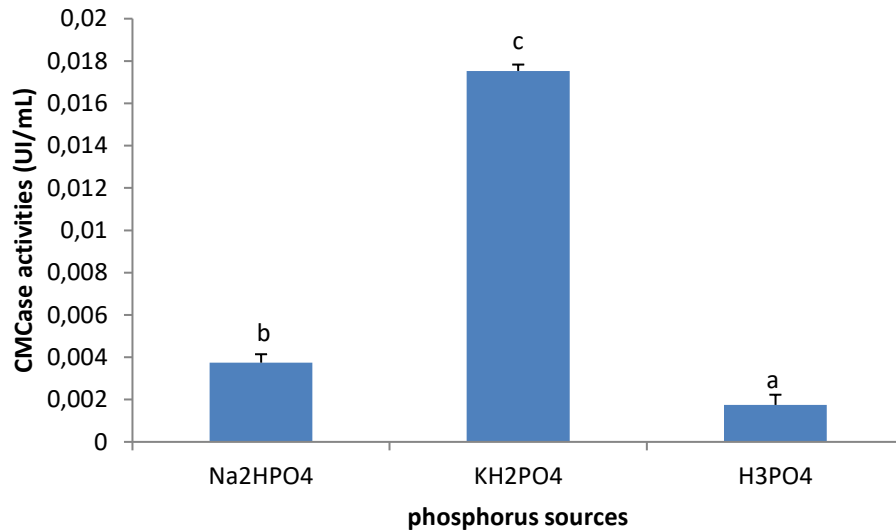


Figure 9. Influence of the source of phosphorus on the enzyme production.

3.4.6 Influence of the KH₂PO₄ concentration on the enzyme production

Figure 10 shows the influence of KH₂PO₄ concentration on enzyme production by isolate B12. The activity of the crude enzyme produced increases rapidly until reaching the maximum value (0.0115 ± 0.0004 IU / mL) at a concentration of 0.6% KH₂PO₄. Beyond these concentrations, activity gradually decreases.

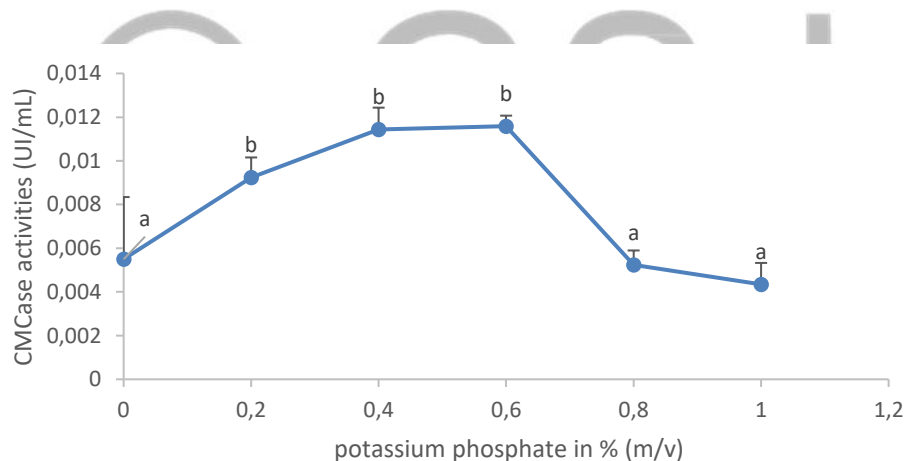


Figure 10. Influence of phosphorus concentration on enzyme production.

3.4.7 Influence of the substrate concentration (CMC) on the enzyme production

Figure 11 shows the influence of substrate concentration on enzyme production by LB12. The activity increases rapidly until reaching the maximum value (0.0250 ± 0.0009 IU/mL) at a concentration of 2% of carboxymethylcellulose (CMC). Beyond this optimal concentration, activity gradually decreases.

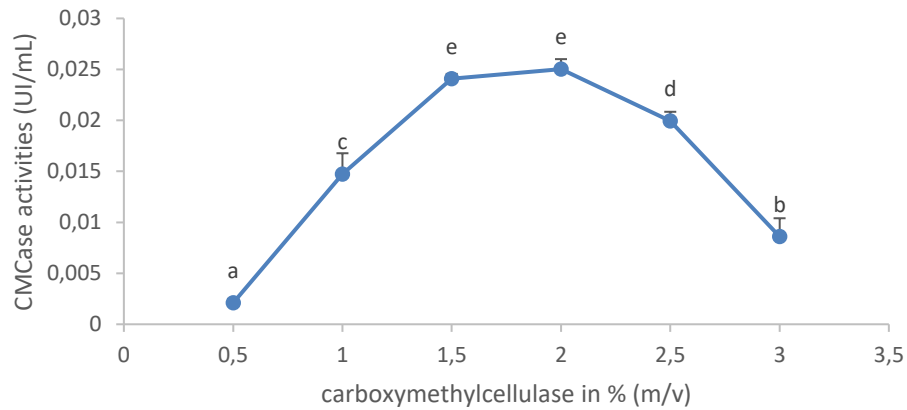


Figure 11. Influence of substrate concentration (CMC) on enzymatic production

3.4.8 Influence of the fermentation time on the enzyme production

Figure 12 shows the influence of fermentation time on enzyme production by isolate B12. There is an increase in enzyme activity until reaching the maximum value (0.0110 ± 0.0004 IU / mL) at 96 hours of fermentation. This activity varies little between 120 and 168 hours.

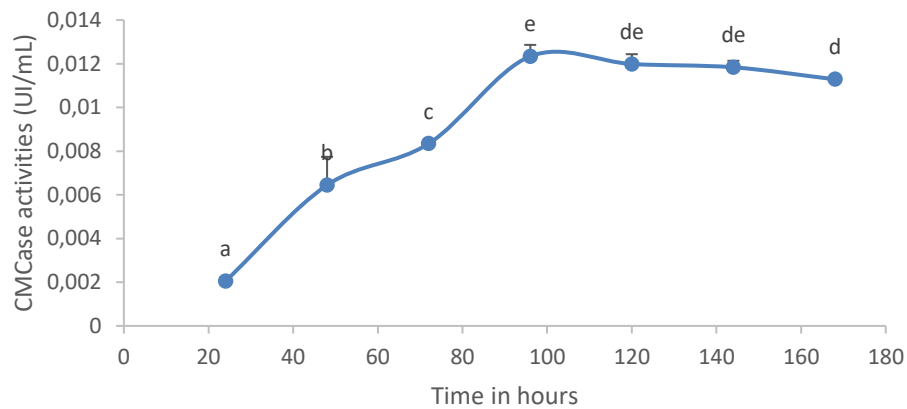


Figure 12. Influence of fermentation time on enzymatic production

4. Discussion

To carry out this work, soil samples were taken in 2 sawmills of over 10 years of age, which enabled us to obtain 53 isolates, ie 18 bacteria and 35 fungi. The sampling site choice focused on the fact that the wood consists of approximately 40-45% cellulose and 25-30% hemicellulose [26]. Thus, sawmill landfills, discharges being mainly made of wood chips, offer microorganisms cellulose and hemicellulose as the main sources of carbon. Therefore, only microorganisms capable of hydrolyzing cellulose or hemicellulose will adapt to this medium, but the adaptability of microorganisms increases over time. Therefore, the older the sawmill the more efficient the microorganisms will be that will adapt to it.

Isolates obtained from the isolation were then subjected to a screening which consisted of determining the enzymatic activity according to three tests carried out successively. The first test is that of determining the degree of viscosity reduction of the fermentation medium. This test enabled us to preselect 16 isolates those whose were greater than or equal to 90%. These 16 isolates were then subjected to the CMCase and APCase activity assay, which enabled us to select the isolate B12 as being the best cellulase producer with a production of 0.0265 ± 0.0016 IU/mL for CMCase activity: and 0.0113 ± 0.0014 IU/mL for APCase activity. It was found that for most isolates, CMCase activity was greater than APCase activity and the two activities evolve in correlation at a rate of 93.73%. Similar results have been reported by [24] when she determining the cellulolytic activity of the bacterial isolates from termite stomach.

The optimum pH obtained at the end of the study of the influence of the initial pH of the medium on the production of cellulase was 7. Above this pH, the production of the enzyme decreases. This variation could be due to the fact that the initial pH of the medium has a significant effect on microbial growth, membrane permeability as well as on the biosynthesis and stability of the enzyme ([20] ; [27]). Similar results have been reported by [15] who establish that most of the cellulases of the microorganisms studied exhibit pH activity for values between 3 and 7, but the pH optima vary depending on the strain microbial.

Regarding the influence of temperature, 45°C is the optimum temperature for cellulase production for isolate B12. In a similar study, maximum cellulase production was observed at 40 ° C for *A. terreus* according to [2] and 37 ° C for *P. fellutanum* according to [14]. The incubation temperature plays an important role in the metabolic activities of microorganisms and the optimum temperature for cellulase production depends on the nature of the microbial strain [21].

The influence of different nitrogen sources on cellulase production shows that casein is the best source of nitrogen for isolate B12. This difference in production can be explained by the fact that B12 would better metabolize caseinic proteins for its nitrogen needs, hence a preference for casein over others, this could be explained by the presence of protease in the enzymatic pool of isolate B12. This result is different from that of [29] who indicated that the nitrogen source should be inorganic for best results. The difference in enzymatic yields in the different studies could be due to differences in cultural practices and different strains of microorganisms used [5].

Regarding the influence of casein concentration, 2.5% is the optimal concentration of cellulosic production. This high concentration of casein could lead us to believe that the action of the protease potentiates that of the cellulase. In addition, nitrogen is important for the metabolism of cells, and the production of proteins. At low nitrogen concentrations, cell metabolism is slowed down, resulting in low protein production and therefore low enzymatic activity. Likewise, the high nitrogen concentrations lead to saturation of the medium with organic compounds, which intoxicates the microbial cells. These observations are in agreement with those found by [7].

Phosphorus is also an important nutrient for cell division because it is part of the composition of nucleic acids. Thus, following the search for the best source of phosphorus for B12, KH_2PO_4 was the best. In addition, the study of the influence of its concentration on cellulase production showed that 0.5% is the optimal concentration of KH_2PO_4 for B12. B12 isolate would therefore have an ease to metabolize KH_2PO_4 to extract its phosphorus compared to other sources. Phosphorus is present in nucleic acids and is used in many enzymatic reactions. It allows the recovery, accumulation and distribution of energy in the bacteria. It is incorporated as an inorganic phosphate.

The influence of the substrate concentration has shown us that 2% CMC is the optimal concentration. The substrate at an optimal concentration is important for cell multiplication because it is a source of carbon. Increasing the concentration of the substrate beyond the optimum can lead to saturation which reduces the formation of enzymes [16].

The influence of time on the production of cellulase shows us that 96 hours is the optimum time of cellulase production, beyond this time the activity is more or less happy this can be caused by the effect of cellobiose which is known to inhibit both endoglucanase and β -glucosidase [13]. This result is in agreement with those reported by [1] who also found 96 h as the optimal cellulase production time from *A. humicola* and *A. niger*, respectively.

5. Conclusion

This study shows that 53 isolates were obtained from 8 samples taken in the two sawmills, 4 per site. Isolate B12 was found to be the best cellulase producer among the 53 with an endoglucanase activity of 0.026 ± 0.0016 IU/ml and APF activity of 0.0113 ± 0.001 4IU / ml. It would be a gram negative catalase positive shell and grows best in a medium containing 2% carboxymethylcellulose, pH 7, temperature 45°C, casein 2.5% (m/v) and dipotassium phosphate 0.6% (m/v). Also we can by this study prove that sawmills are sustainable sources of microorganismes witch can be used for industrial purposes.

6. Acknowledgments:

Authors would like to thank the Biochemistry laboratory at the University of Douala, which provided us with the technical platform necessary for the smooth running of this work. We would also like to thank our collaborators from the biochemistry laboratory at the University of Ngaoundéré who provided us with some assay reagents. For this study special thanks to M. Tobit Pascal of the biochemistry laboratory, my daily partner who helped us in editing this paper.

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