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Monitoring of Cellulase and Biosurfactant Production in Bacteria Isolated from the Likouala Peat Bog (Republic of Congo)

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Abstract The production of cellulases and biosurfactants of isolated bacteria from the Likouala peat bog (Congo-Brazzaville) was studied. Growth and production of cellulases and biosurfactants were monitored by conventional techniques on Mossel and TSB media enriched with petroleum or vegetable oil. Correlation coefficients between cellulases and biosurfactants production were calculated with Microsoft Excel 2016 software. The results showed optical densities from 0.634 to 1; diameters of translucent halos from 1.4 to 5.8cm and emulsification indices of isolates from 0 to 80%. The production time of cellulases and biosurfactants was 24 to 96 h with an optimum at 72h for biosurfactants depending on the isolate. The isolate correlation index were: r=0.74 and r=0.53 for RE 20; r=0.87 and r=0.60 for RE 21 and r=0.96 and 0.91 for RE 24 on Mossel; r=0.77 and r=0.61 for BV1; r=0.96 and r=0.95 for BV2; r=0.95 and r=0.52 for BV3 and r=0.92 and r=0.83 for BV4 on TSB enriched with petroleum; r=0.95 and r=0.86 for BV6; 0.54 and 0.57 for BV7 and r=0.84 and r=0.95 for BV9 on TSB enriched with vegetable oil respectively for the production of cellulases and biosurfactants. The Likouala peat bog area is rich in hydrocarbonoclast bacteria that produce cellulases and biosurfactants from peat.

Keywords: *biosurfactant, cellulase, correlation, bacteria, bog*

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1. Introduction

The Likouala peat lands are located in the Congo Basin where they extend over an estimated area of 145,500 km^2 and store approximately 30.6 pentagrams of carbon [1]. Peats form from swampy forest vegetation dominated by leaf litter and the palm species *Raphia laurentii* and *Raphia hookeri* that occupy abandoned river channels [1,2]. In this ecosystem, microorganisms are actors of the degradation of organic matter of plant origin (peat). They influence not only the storage and release of carbon but are also likely to produce cellulases and biosurfactants that can be used in several industries, in bioremediation and in depollution. In recent years, there has been a growing demand for enzymes of industrial interest and cellulases occupy the third place in the world due to their multiple applications in the pulp and paper industry, textiles, biofuels, extracting juice from fruits and vegetables. Also, these enzymes have become the target in academic and industrial research [3]. Several studies have shown that they are secreted by a wide range of microorganisms, including filamentous fungi, bacteria and yeasts [4,5]. Some studies indicate a close relationship between the production of cellulases and that of biosurfactants from organic matter by bacteria of the Bacillus and Pseudomonas genera [4,6,7]. Biosurfactants are molecules that respect the environment, are less toxic and biodegradable. They are endowed with antimicrobial, antiviral, antitumor, anti-adhesive, anti-biofilm potential. Some may be active at extreme pH, temperature and salinity [8]. Moreover, biosurfactants have a large repertoire that allows them to degrade a wide range of organic pollutants [9]. The prospects of biosurfactants have great potential due to their applications in the petroleum industry [10]. It is in order to appreciate the bacterial activities in the evolution of the quality of peatlands, their impact on the environment and to establish a correlation between the production of cellulases and biosurfactants that the present work was initiated.

2. Material and Methods

2.1. Sample Collection

The study was carried out using 10 isolates obtained from nine peat samples taken by coring in the Likouala peat bog and kindly provided by Morabandza and al, (2022). These were: 9 (nine) isolates of the genus *Bacillus*, including 3 (three) obtained on Mossel medium, 6 (six) on TSB medium enriched with petroleum or vegetable oil and 1 (one) *Enterobacter* sp on TSB medium enriched with vegetable oil.

2.2. Isolation of Hydrocarbonoclast Bacteria

Bacteria were isolated from a positive enrichment culture. A series of 8 successive 1/10th dilutions with sterile distilled water was carried out for surface inoculation on Petri dishes containing TSB agar enriched with petroleum or vegetable oil. The inoculated dishes were incubated at 37°C in an oven for 3 days. Separate formed colonies were purified by the successive depletion method on liquid TSB medium still containing the target substrates as a carbon source [12]. The protocol is thus repeated until pure colonies are obtained. The purified bacteria were checked by microscopic observations (fresh state and Gram stain) and biochemical test (catalase test).

2.3. Optimization of Cellulase and Biosurfactant Production

In order to study the production of cellulases and biosurfactants, cultures were carried out on liquid TSB medium enriched with petroleum and vegetable oil respectively 3 isolates on TSB enriched with 1% vegetable oil; 4 isolates on TSB enriched with 1% petroleum and 3 isolates on liquid TSB. After 7 days of incubation at t0; t1; t2; t3; t4; t5; t6 and t7 at 37°C, 5ml of inoculum containing the culture were taken every 24 hours (0, 24h, 48h, 72h, 96h, 120h and 144h). For each sample, 2ml of the inoculum were used to measure the optical density at 600nm using a Zuzi 4211/50 type spectrophotometer. 1ml to assess cellulase production and 2ml to perform the emulsion test.

2.4. Cellulase Production

Masses of 0.5g of cellulose and 1.5g of agar were weighed and dissolved in 100ml of distilled water. The mixture was sterilized in an autoclave at 121°C for 15mn and then poured into Petri dishes. Wells were then prepared and 100µl of suspension of each Overnight culture isolate placed and the dishes incubated for 48hours at 37°C in an oven. The revelation was made with the Lugol. The presence of a translucent zone around the well indicates the production of cellulase by the strain; the diameter of each translucent halo is measured [13].

2.5. Emulsion Test

To perform the biosurfactant emulsion test, 2ml of petroleum and oil were added respectively with 2ml of the culture in test tubes, then vortexed for 2 minutes and allowed to stand then incubate for 24 hours and, emulsification index was calculated according to the relationship below.

$$E_{24} = \frac{He}{Ht} \times 100$$

E_{24}: Emulsification activity after 24h; He: Height of the emulsion formed; Ht: Total height of the mixture [14].

2.6. Results Analysis

The statistical results were represented, illustrated and analyzed by Microsoft Excel 2016 software.

3. Results

3.1. Number of Isolates Obtained and Characterization of Isolates on Mossel Medium

Figure 1 represents the growth kinetics on Mossel of isolates RE20, RE21 and RE24 as a function of time. The variation of the obtained curves presents the same pace, the same phases with different amplitudes at the level of the 3 isolates. There is an exponential growth phase, a slowing phase, a stationary phase and finally a declining phase. At the level of isolates RE20, RE21 and RE24, from t0 to t1 (0 to 24h), the increase in growth is progressive and proportional to time; which corresponds to the exponential phase of growth. From t1 to t2 (24 to 48h) it is the slowing down phase. From t2 to t4 (48 to 96 h), despite the increase in time, a stabilization of growth is observed, thus corresponding to the stationary phase. Beyond 96h, the growth (D.O) becomes inversely proportional to the time, it is the phase of decline.

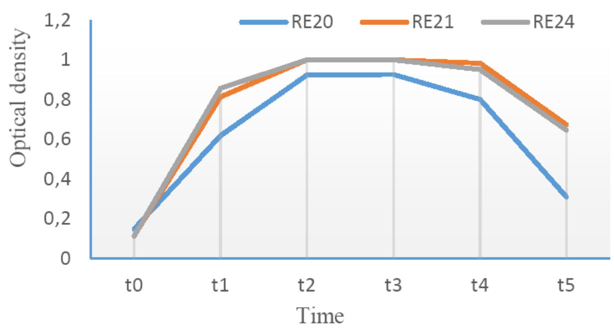

Figure 1. Cinetic growing curve of Mossel isolates

3.2. Production of Cellulases and Biosurfactants of Isolates on Mossel

Cellulase production and emulsification index (percentage) indicating biosurfactant production in three Mossel isolates RE20, RE21 and RE24 are time and isolate dependent. Figure 2-a provides information on cellulase production in RE20, RE21 and RE24. At RE20, production decreases from t0 to t1 (0 to 24h), it remains stable from t1 to t3 (24 to 72h) and reaches its maximum at t4 (96 h). Beyond t4, the production decreases to reach its minimum value at t5 (120h). At RE24, from t0 to t1 (0 to 24h) an average production is observed; this production becomes stable from t1 to t3 and reaches a peak at t4 (96h). Beyond t4, it slows down until t5 (120h). Whereas, in RE21, from t0 to t1 (0 to 24h), there is an absence of cellulase production. From t1 to t3 (24 to 72h), the production increases as a function of time and reaches its optimum at t4 (96h). Beyond that, it decreases and becomes average and vanishes at t5 (120h). Figure 2-b indicates the evolution of the percentage of the emulsification index over time. Biosurfactant production is time and isolate dependent. In the isolates, the curves show the same shape. From t0 to t1 (0 to 24h) production is low and increases over time at t2 (48h). From t2 to t5 (48 to 120h), at RE20 and RE24 production is proportional to the increase in time and continues to increase at t5 (120h). Whereas, at RE21, it reaches its optimum at t2 (48h) and vanishes at t5 (120h).

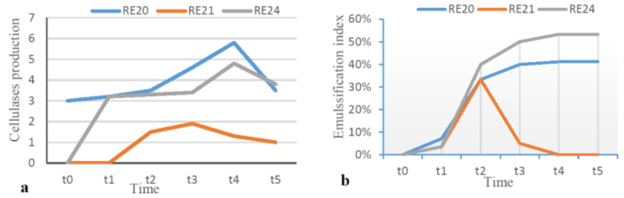

Figure 2. Cellulase production and emulsification index of Mossel isolates

3.3. Growth of Isolates on Petroleum-Enriched TSB

Figure 3 shows the growth kinetics in four isolates cultured on TSB medium enriched with petroleum Bv1, Bv2, Bv3 and Bv4 as a function of time. These curves present the same paces with different amplitudes: an exponential growth phase, a slowing down phase, a stationary phase and finally a declining phase. In Bv2, Bv1, Bv3 and Bv4 from t0 to t1 (0 to 24h) there is a simultaneous increase in growth and time, this corresponds to the exponential phase, from t1 to t2 (24 to 48h) it is the slowdown phase. From t2 to t5 (48 to 120h); then, we observe a stabilization of the D.O expressing the stabilization of growth (D.O): this is the stationary phase. Beyond t5 (120h), growth (D.O) becomes inversely proportional to time, which corresponds to the decline phase.

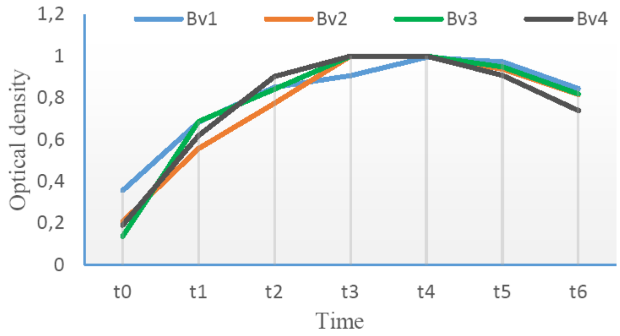

Figure 3. Cinetic growing profil of isolates in to petroleum medium

3.4. Production of Cellulases and Biosurfactants of Isolates on Petroleum-Enriched TSB

In the four (04) isolates, cellulase production is variable depending on time and the isolate depends on time (Figure 4-a). In Bv1 and Bv4 the production is average from t0 to t4 (0 to 96h) and reaches a peak at t5 (120h). Beyond that, it decreases and is canceled at t6 (144h). In Bv2, from t0 to t2 (0 to 48h) an average production is observed, then it increases and reaches its optimum at t3 (72h) in proportion to time. Beyond t3, it slows down and becomes inversely proportional to time and so much to cancel out at t6 (144h). In Bv3, from t0 to t3 (0 to 76h) the production is average, reaching a peak at t4 and decreasing at t6 (144h). The emulsification index indicating the production of biosurfactants (Figure 4-b), in Bv1, Bv2, Bv3 and Bv4 cultured in the presence of oil depends on time. The curves show the same shape. From t0 to t1 (0 to 24h) the production of biosurfactants is average and increases from t2 (48h). From t2 to t5 (48 to 120h), in Bv2, Bv3 and Bv4 the production is proportional to the increase in time and continues to increase until t6 (144h). While in Bv1, it reaches a peak at t3 (72h) and becomes inversely proportional to time until t6 (144h) beyond t3 (72h).

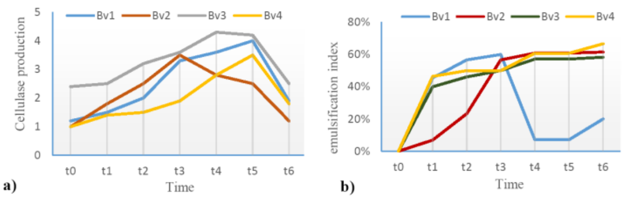

Figure 4. Cellulase production and emulsification index of isolates in TSB petroleum supplemented

3.5. Growth of Isolates on TSB Enriched With Vegetable Oil

Figure 5 illustrates the growth curves in three (03) isolates cultured in the presence of vegetable oil Bv6, Bv7 and Bv9 as a function of time. From t0 to t1 (0 to 24h), an exponential growth phase is observed; from t1 to t2 (24 to 48h) is the phase of slowing growth; from t2 to t5 (48 to 120h) it is the stationary phase, there is a stabilization of the D.O thus expressing the stabilization of the growth (D.O) and finally from t5 to t6 (120 to 144h) the growth

(D.O) becomes inversely proportional to time, which corresponds to the decay phase.

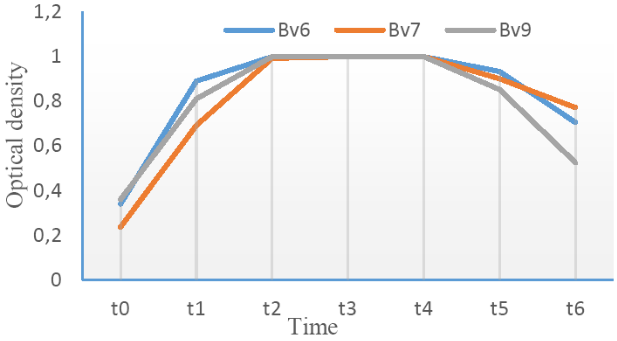

Figure 5. Cinetic growing of isolates in presence of vegetable oil

3.6. Production of Cellulases and Biosurfactants on TSB Enriched With Vegetable Oil

Figure 6-a makes it possible to follow the kinetics of cellulase production in three isolates Bv6, Bv7 and Bv9 cultured in the presence of vegetable oil. Production is variable depending on the isolate and time. From t0 to t1 (0 to 24h) in the three isolates the production is average and becomes maximum at t1 (24h). Beyond t1 in Bv6 and Bv7, production decreases and remains stable until t6 (144h). While at Bv9 beyond, it slows down and becomes inversely proportional to time to cancel out at t6 (144h). The emulsification index of these BV6, BV7 and BV9 isolates likewise varies with time (Figure 6-b). From t0 to t2 (0 to 24h) production remains average and from t2 (48h), it increases sharply. From t2 to t5 (48 to 120h), in BV6 and BV9, the emulsification index is proportional to the increase in time and drops at t6 (144h). Whereas in BV7, it reaches a peak at t2 (48h) and becomes inversely proportional to time beyond t2 to vanish at t4 (96h).

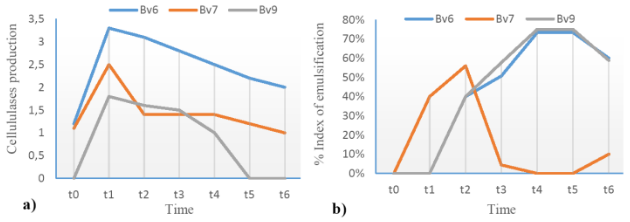

Figure 6. Cellulase production (a) and emulsification index (b) of isolates cultivating in vegetable oil

3.7. Correlation between Growth and Biosurfactant Production

3.7.1. Case of Isolates Grown on Mossel

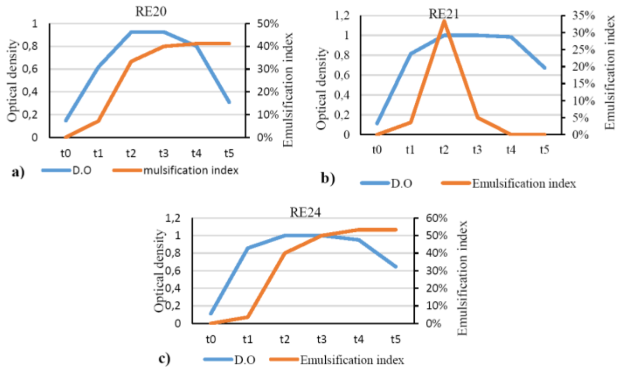

Figure 7. Correlation beetween biosurrfactants production and cinetic growing of Mossel isolates : a) RE20 ; b) RE21 ; c) RE24

Figure 7 shows the relationship between growth (D.O) and biosurfactant production in RE20, RE21 and RE24. A gradual increase in the emulsification index was observed with the D.O, which increased with time up to t2 (48h) in the three strains. In RE21, the emulsification index reaches a peak at 48h (t2) then decreases and tends to be canceled at t4 (96h) as well as the D.O. However in RE20 and RE24, the D.O (growth) and the emulsification index increases simultaneously up to t3 (72h). At t4 (96h), growth declines while the emulsification index remains stable.

3.7.2. Case of Isolates Grown on TSB Enriched with Petroleum

Figure 8 illustrates the relationship between the O.D (growth) and the emulsification index in isolates Bv1, Bv2, Bv3 and Bv4 cultured in the presence of oil. A concomitant increase is observed between the emulsification index and the O.D in BV2, BV3 and BV4 up to t4 (96h). Beyond 96h, the index remains constant while the O.D decreases. In BV1, it gradually increases with the O.D and reaches a peak after t3 (72h) then, the emulsification index decreases and tends to be canceled at t4 (96h) while the O.D continues to increase and declines from of t5 (120h).

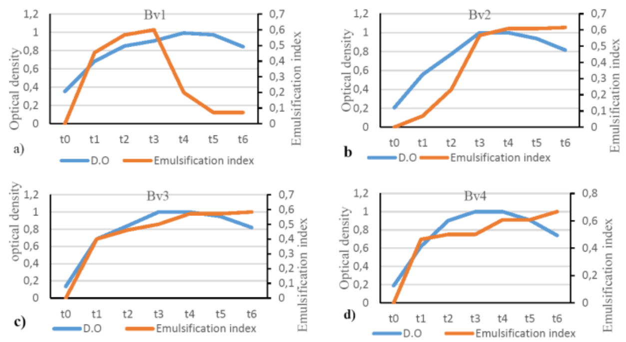

Figure 8. Correlation beetween biosurrfactants production and cinetic growing of petroleum isolates a) Bv1 ; b) Bv2 ; c) Bv3 ; d) Bv4

3.7.3. Case of Isolates Grown on TSB Enriched with Peanut Oil

Figure 9 presents the relationship between the D.O (growth) and the emulsification index in isolates Bv6, Bv7 and Bv9 cultured in the presence of peanut oil. In Bv6, Bv7 and Bv9 the index increases simultaneously with the optical density and reaches a peak after 72h (t3). 96h (t4) later, it remains constant while the O.D decreases. All the curves present the same pace, the increase in the index becomes inversely proportional to the O.D which continues to increase to decrease after 120h (t5).

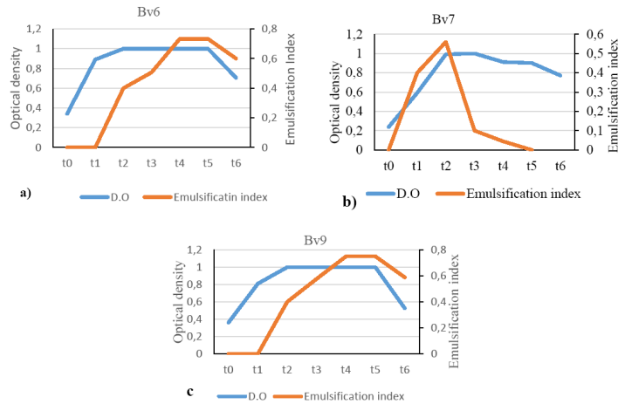

Figure 9. Correlation beetween biosurrfactants production and cinetic growing of vegetable oil isolates a) BV6 ; b) BV7 ; c) BV9.

3.8. Correlation between Cellulase (PC) and Biosurfactant (PB) Production

3.8.1. Case of Isolates on Mossel Medium

Figure 10 shows the monitoring of the production of cellulases (PC) and of biosurfactants (PB) of isolates RE20, RE21 and RE24 on Mossel medium. The cellulase production and emulsification equation is of the type $Y=ax^2+bx+c$. With the RE20 isolate, the production of cellulase increases from the 1st to the 6th day, the emulsification index is only up to the 4th day, with r=0.53 for the emulsification index and r =0.74 for cellulase production. It is then decoupled from the production of cellulase and decreases to reach the minimum value on the 6th day (Figure 10-a). With the RE21 isolate (Figure 10-b), the production of cellulase increases from the 1st to the 4th day, that of emulsifiers is only up to the 3rd day with r=0.87 and r= 0.60 respectively then, it decreases to reach its minimum value on the 6th day. The decrease in the rate of emulsification corresponds to that of the production of cellulase. In the RE24 isolate (Figure 10-c), r=0.96 for the emulsification index and r=0.91 for the production of cellulase. The emulsification index is strongly linked to the production of cellulase until the 5th day. The decrease in cellulase production has little impact on that of biosurfactants.

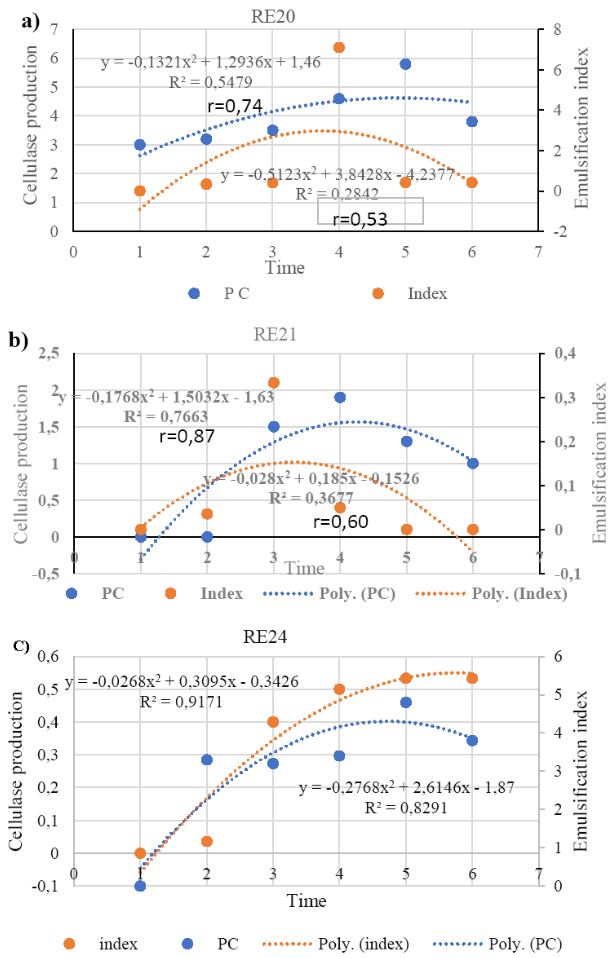

Figure 10. Correlation beetween emulsification index and cellulase production of Mossel isolates a) RE20 ; b) chez RE21 ; c) chez RE24

3.8.2. Case of Isolates Grown on TSB Enriched with Petroleum

The cellulase production and emulsification equation is of the type $Y=ax^2+bx+c$. In the Bv1 isolate, cellulase production increases from the 1st to the 6th day with r=0.77; that of biosurfactant is only observed up to the 4th day with r=0.61 (Figure 11-a). It then decreases to reach its minimum value on the 6th day. Figure 11-b shows that with the Bv2 isolate, the production of biosurfactants increases from the 1st to the 7th day even when that of cellulase decreases from the 4th day with r=0.96 and r=0.95 respectively. Figure 11-c, with the Bv3 isolate on the other hand, shows a concomitant increase in the production of cellulase (r=0.95) and biosurfactants (r=0.52) until the 5th day. The decrease in the production of biosurfactants corresponds to that of the production of cellulase. Figure 11-d however reveals that the production of biosurfactants (r=0.92) is strongly linked to that of cellulase (r=0.83) in the Bv4 isolate until the 5th day. The decrease in cellulase production has little impact on that of biosurfactants.

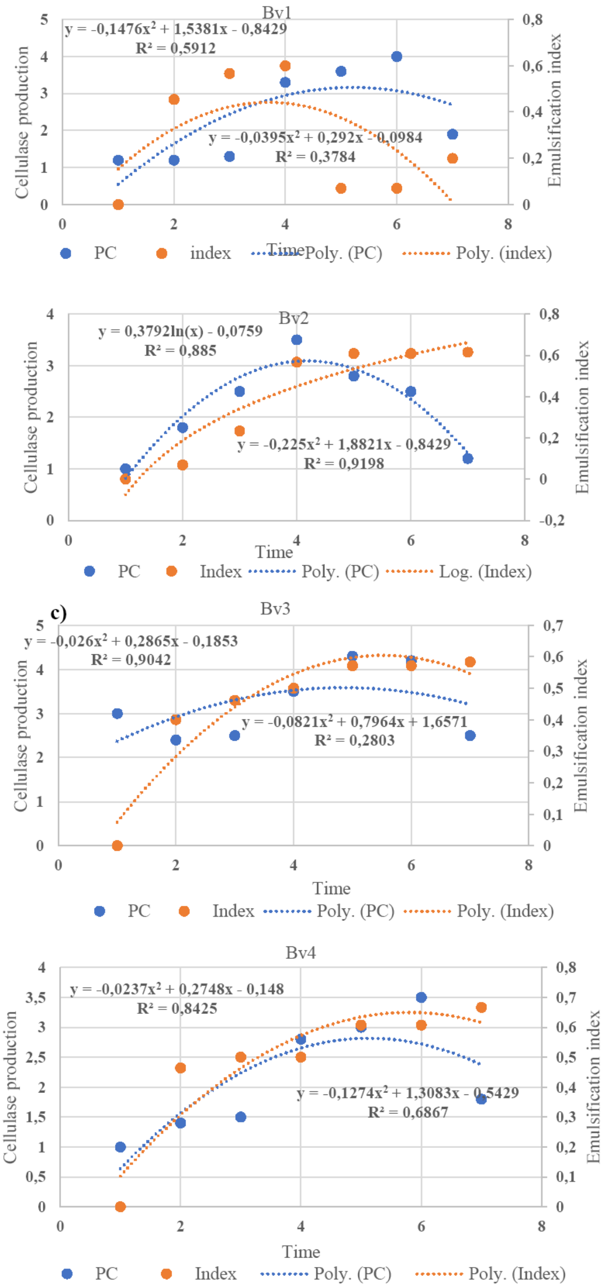

Figure 11. Correlation beetween emulsification index and cellulase production of isolates a) Bv1 ; b) Bv2 ; c) Bv3 ; d) Bv4

3.8.3. Case of Isolates Grown on TSB Enriched with Peanut Oil

Figure 12 indicates the monitoring of the production of cellulases and biosurfactants on TSB medium of isolates Bv6, Bv7 and Bv9. Figure 12-a shows a simultaneous increase in the production of cellulase and biosurfactants from the 1st to the 4th day in the Bv6 isolate with r=0.95 and 0.86 respectively. While the production of cellulase decreases, that of biosurfactants continues to increase until the 6th day to decrease from the 7th day. With the Bv7 isolate (Figure 12-b), the production of biosurfactants and cellulases evolves concomitantly until the 4th day r=54 and r=57 respectively. Beyond this period, a decrease in the production of cellulase as well as that of biosurfactants was observed. In the Bv9 isolate (Figure 12-c), as in the previous isolates, the production of biosurfactants increases consecutively to that of cellulases with time until the 4th day (Figure 12-c). This production of cellulases subsequently decreases and becomes inversely proportional to that of biosurfactants. The evolution of the two processes show very high correlation coefficients respectively r=0.95 for the production of cellulase and r=0.84 for the biosurfactants.

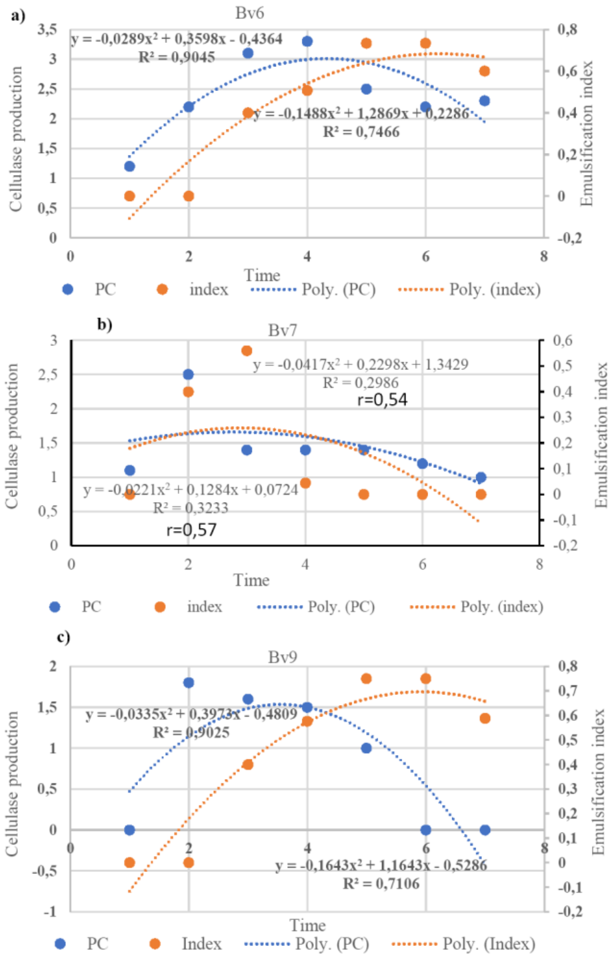

Figure 12. Correlation beetween emulsification index and cellulase production of isolates a) Bv6 ; b) Bv7 ; c) Bv9

4. Discussion

The cellulase and biosurfactant industry has experienced remarkable growth in recent decades. However, their large-scale production remains a challenge from the economic point of view and the requirements (time required for fermentation, target market, properties and purification processes) for a viable and profitable production [8]. This work aimed to study the production of cellulases and biosurfactants in bacteria isolated from the Likouala peat bog in Congo-Brazzaville. The results of the experiment revealed that the growth curves in the 10 isolates studied present the same paces: an exponential phase, a slowing phase, a stationary phase and that of growth decline with, however, an absence of a latency phase. This absence would certainly be explained by the interval time of the measurements of the optical densities since, during our experiment, the measurements were carried out every 24h. Optimal growth in the ten (10) isolates studied was observed after 48h (t2). These same observations were made by Nguimbi et al. (2014) and Soloka et al. (2017) with *Bacillus* strains isolated from Ntoba mbodi in Congo-Brazzaville. However, under our experimental conditions, it is after 72 hours that the ten isolates produce cellulases with large diameters ranging from 1.4 to 5.8cm and emulsification index from 0 to 80%, i.e. beyond 48h which is the optimal growth time. This fact suggests that cellulases and biosurfactants production does not depend on the growth of the isolates. These are two distinct but related phenomena. Here, the production is average during the exponential phase then gradually, it increases and reaches its maximum at the stationary phase depending on the isolate. In isolates RE20, RE21, RE24, Bv6 and Bv9 during the exponential phase there is no production, it begins after 48h corresponding to the stationary phase and gradually accumulates to reach a maximum around 96 and 120h. Whereas in isolates Bv1, Bv2, Bv3, Bv4 and Bv7, the production starts from the exponential phase and reaches its maximum at the stationary phase. These results clearly show that the optimum production of biosurfactants is after 48h, thus suggesting a correlation with the production of cellulase by the isolates studied. Other authors have shown the correlation between the production of cellulases and biosurfactants in *Bacillus cereus* 11778 [4,7].

In this study, this relationship was determined by calculating the correlation coefficients between the production of cellulase and the emulsification index. A critical analysis of the values of the correlation coefficients shows that it would depend on the performance of the isolate and also on the experimental culture medium. In the case of isolates cultured on Mossel medium, the values of the coefficients (r=0.43 and r=0.74) of RE20 indicate that the production of cellulase does not influence that of biosurfactants. Unlike RE21 (r=0.87 and r=0.60) or, the decrease in the emulsification index corresponds to that of cellulases; as well as with RE24 (r=0.96 and 0.91) where the production of the two substances is strongly dependent on the 5th day of experimentation and the drop in cellulase production impacts the increase in the rate of emulsification. In the case of isolates cultured on TSB enriched with petroleum, with Bv2, it is noted that the production of biosurfactants increases from the 1st to the 7th day even when the production of cellulase decreases from the 4th day. This is explained by the fact that the cellulase production values remain high compared to those of Bv1 where no correlation is observed. Unlike the Bv3 and Bv4 isolates, a strong concomitant of cellulase production and emulsification is observed until the 5th day. The decrease in the production of cellulases has little impact on that of biosurfactants. The case of isolates cultured on TSB enriched with peanut oil presents a situation rather opposite to that of the first two media. The production of biosurfactants remains dependent on that of cellulase. With Bv6 (r=0.95 and r=0.86) a simultaneous increase in the production of cellulase and in the rate of emulsification is observed until the 4th day. As cellulase production declines, the rate of emulsification continues to increase. The decrease in the rate is observed from the 7th day, this is explained by the fact that the values of cellulase production remain high compared to those of the emulsification index. With Bv7 (r=0.54 and r=0.57) similar correlation coefficients, the rate of emulsification and the production of cellulase evolve concomitantly until the 4th day. Beyond this period, a decrease in both cellulase production and the emulsification index is observed. As in the previous isolates, in Bv9 (r= 0.95 and r=0.84), the emulsification index increases over time until the 4th day. This increase is consecutive to that of the production of cellulase. Then, it decreases and becomes inversely proportional to the rate of emulsification. The equations of the evolution of the two processes show very high correlation coefficients. With regard to the correlation coefficients, the Bv2 isolate on TSB enriched with petroleum presents a better production (r=0.96 and r=0.95) followed by RE24 (r=0.96 and r=0.91) on Mossel ; isolate Bv6 on TSB enriched with petroleum (r=0.95 and r=0.86) and Bv9 (r=0.84 and r=0.95) on TSB enriched with peanut oil whereas the Bv7 isolate (r=0.54 and r=0.57) on TSB enriched with peanut oil shows the lowest production of cellulase and biosurfactants. Our results are similar to those of Khelil (2017) who also noticed a drop in surfactant production in the medium after 72 hours with the drop in the amount of cellulase in the experimental medium. They are also similar to those of Zheng et al. (2021) who reported that biosurfactants such as rhamnolipids can improve and increase the yield of xylanase and cellulase. At peak cellulase production, rhamnolipid and Tween 80 increased cellulase activities by 20-50%.

5. Conclusion

Ultimately, this study showed that optical densities of 0.634 to 1; diameters of 1.4 to 5.8cm and emulsification indices of the isolates of 0 to 80%. The production time of cellulases and biosurfactants was 24 to 96h with an optimum at 72h for biosurfactnts depending on the isolate. Isolate Bv2 on petroleum-enriched TSB shows better production (r=0.96 and r=0.95) followed by RE24 (r=0.96 and r=0.91) on Mossel ; isolate Bv6 on TSB enriched with petroleum (r=0.95 and r=0.86) and Bv9 (r=0.84 and r=0.95) on TSB enriched with peanut oil whereas the Bv7 isolate (r=0.54 and r=0.57) on TSB enriched with peanut oil shows the lowest production of cellulase and biosurfactants. The Likouala peat bog area is rich in hydrocarbonoclast bacteria that produce cellulases and biosurfactants from peat.

Statement of Competing Interests

The authors declare no conflict of interest

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