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Prime Enzymatic Exocellular Background of *Lysinibacillus louembei*

Moïse Doria Kaya-Ongoto[1,2], Christian Aimé Kayath[1,2*], Alain Brice Vouidibio Mbozo[1], Gyna Mobandolaka Mitoko[1,2], Sandra Paola Elenga Wilson[1,2], Duchel Jeanedvi Kinouani Kinavouidi[1,2], Etienne Nguimbi[1,2]

[1]Laboratoire de Biologie Cellulaire et Moléculaire (BCM), Faculté des Sciences et Techniques, Université Marien NGOUABI, Brazzaville, République du Congo
[2]Institut National de Recherche en Sciences Exactes et Naturelles (IRSEN), Avenue de l'Auberge Gascogne, Brazzaville, Congo
Email: *chriskayath@yahoo.fr

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Abstract

Many high-reading journals reject several studies based on the basic aspects of microbiology by forgetting that this could open doors and windows of great scientific discoveries. A new spore-forming bacteria species called *Lysinibacillus louembei* has been previously discovered in our research unit. This new study aims to assess a real enzymatic machine produced with such bacteria. Using agarose mixed with half skimmed milk, LB medium supplemented with tween 20, Olive oil, egg yolk, cellulose, casein, pectin and starch, we showed that *L. louembei* has lipolytic, proteolytic (2.8 cm ± 0.1), cellulolytic, amylolytic pectinolytic activities, with percentage ranging from 30% to 80%. This species is able to secrete lipase, thermostable protease up to 60˚C. Cellulase, pectinase and amylase are secreted with more stability between 30˚C and 50˚C with an optimum at 45˚C. The effect of pH was determined after 24 h of incubation at 37˚C and 50˚C. The proteolytic activity is stable at pH 8 at 50˚C. In addition to this first enzymatic landscape, *L. louembei* is able to degrade and/or tolerate gasoline and/or diesel fuel hydrocarbons in 36 hours. In this work, we also showed that *L. louembei* can produce biosurfactant in the presence of gasoline and SAE140 with the E24 up to 50%.

Keywords

Lysinibacillus louembei, Prime Enzymatic Exocellular, Bacteria

1. Introduction

The genus *Lysinibacillus* is consistently characterized by rod-shaped bacilli that form endospores [1] with an A4*α* (L-Lys–D-Asp) cell-wall peptidoglycan

type [2] [3]. Menaquinone MK-7 is the most predominant respiratory lipoquinone system among the genus *Lysinibacillus*, and major polar lipids found are diphosphatidylglycerol, phosphatidylglycerol and ninhydrin-positive phosphoglycolipid [2]. *L. louembei* is a novel spore-forming bacterium isolated from Ntoba Mbodi which is an alkaline fermented leave of cassava from the Republic of the Congo [4]. By drafting this paper, the genus *Lysinibacillus* contains about thirty species. *L. louembei* was also postulated to produce bacteriocins-like molecules. This novel exciting Gram positive bacterium with unusual phenotypic, biochemical and genotypic characteristics has been tested for the ability to kill pathogenic bacteria such as *Samonella* spp. *Staphylococcus* spp. and *Shigella* spp. Maybe biosurfactants found recently in other bacteria like *L. fusiformis* S9 could easily play this role [5]. Microorganisms that can play the probiotic role and that belong to the genus *Lisinibacillus* are not numerous. The study conducted by Ouoba *et al*. has allowed asserting that these bacteria could be a bright probiotic and prebiotic candidate [4].

Although first studies have been carried out on morphological characterization and some biochemical specificities including probiotic potential and some antibacterial activities, to our close knowledge, no updated study has focused on the production of biomolecules such as extracellular enzymes. In addition through the best of our knowledge few studies have been demonstrated the abilities of *Lisinibacillus* genera with several enzymatic activities including lipolytic, proteolytic, cellulolytic, pectinolytic and amylolytic activities. This study aims to study exocellular enzyme of *L. louembei*, and to assess his role in lipid, protein, cellulose, amylose, pectin degradations.

2. Materials and Methods

2.1. Bacterial Strains

The main biological material used is *Lysinibacillus louembei* strain a bacterium that has been discovered and isolated from Ntoba Mbodi, a fermented cassava leaves in the Republic of Congo [4]. This bacterium is an integral part of our future potential strains for more characterization. *Bacillus* sp. and *B. licheniformis* have been used as control [6]. Lab strains like *E. coli* TOP 10 and BL21 have been used.

2.2. Enzymatic Activities

2.2.1. Determination of Lipolytic Activities

To test lipolytic activities, three substrates have been used including Tween 20, Olive oil and York egg. 10 drops of Tween 20 or olive oil have been aseptically mixed in solid LB medium and Mossel medium with yellow york egg. Three media are then poured into the petri dishes. 10 µL of O.D 0.8 of the supernatant are deposited on the petri dish always containing the on LB medium supplemented with tween 20. O.D value was performed under 600 nm as wavelength. A bacterial colony is spread on the boxes containing the egg yolk and the olive oil. The Petri dishes are incubated at 37˚C for 24 hours. After overnight incubation the

test is positive with the presence of an opaque halo around the bacterial colony.

2.2.2. Determination of Proteolytic Activity

Some *Bacillus* strains were assessed for the ability to secrete proteases in the extracellular environment as described and modified by Kaya-Ongoto *et al*., 2019 [6]. Briefly, 1 g of agarose was weighed and mixed with 100 mL of PBS. The mixture was heated in a microwave for 3 min until agarose was completely dissolved and then cooled in a water bath at 40˚C. Then 10 mL of skim milk was added to the mixture. After homogenization, the mixture was poured into the Petri dishes. Once solidified, wells were carefully and aseptically generated into the gels. A volume of 50 µL of the overnight culture supernatant is deposited in the wells made on the agar medium composed of 1% agarose gel, 0.01 M PBS, pH 7.4 and skimmed milk. The Petri dishes are incubated at 37˚C for 24 hours. The presence of proteolytic activity is detected by a clear halo around colonies indicating hydrolysis of casein. The halo diameters were measured.

2.2.3. The Influence of Temperature and pH on Proteolytic Enzyme Production

In order to assess the influence of temperature, 5 mL LB broth *L. louembei* cultures were run at different temperatures including 25˚C, 30˚C, 35˚C, 37˚C, 40˚C, 45˚C, 50˚C and 55˚C, the optical density (OD) was measured by spectrophotometer at 600 nm, the microbial suspension was centrifuged at 10,000 rpm for 10 min at room temperature. 50 µL were placed in wells of the milk agarose medium contained in the petri dishes. All the dishes are incubated at their respective overnight culture temperatures. The diameters have been measured.

To know the influence of pH on the production of extracellular proteases an overnight culture of 5 mL was performed in LB Broth at different pH (7, 7.5, 8, 8.5, 9, 9.5 and 10). Incubation of cultures at different pH was done at 37˚C for 24 hours. The cultures were centrifuged and the different pH supernatant was recovered to deposit 50 µL in each well generated in the milk agarose plates. Then the Petri dishes were incubated at 37˚C and 50˚C and the diameters were measured as well.

2.2.4. Amylolytic, Cellulolytic and Pectinolytic Activities

To assess the amlylolytic, cellulolytic, pectinolytic activity of *L. louembei*, an overnight culture was carried out on a Petri dishes containing LB medium for the purpose of well isolated colonies. Then a young bacterial colony was deposited on the surface on solid LB medium separately added to 1% of starch, 0.5% of cellulose and 0.5% of pectin. The Petri dishes are incubated 48 h to 72 h, at 37˚C. The revelation is made with Lugol. A test is positive if there is a clear halo around the colony corresponding to a lysis range.

2.2.5. Degradation and Tolerance to Hydrocarbon and Evaluation of Emulsion Index (E24)

The capacity of *L. louembei* to degrade and/or to tolerate hydrocarbons was

evaluated according to the capacity to utilize gasoline, diesel fuel or SAE 140 hydrocarbons in Bushnell-Haas (BH) minimal salts media as previously described by Kayath *et al*., 2019 [7]. Briefly, *L. louembei* would be cultivated for 14 days at 37˚C by using an adapted Bushnell-Haas (BH) minimal salts media composed of 10 g/L NaCl, 0.29 g/L KCl,; 0.42 g/L $MgSO_4 \cdot 7H_2O$, 0.83 g/L KH_2PO_4, 0.42 g/L NH_4SO_4, 1.25 g/L K_2HPO_4 [8]. The medium was adjusted to pH 7.2 and supplemented with gasoline, diesel fuel or SAE140 (1 mL for 300 mL of medium). This experiment was done in triplicate.

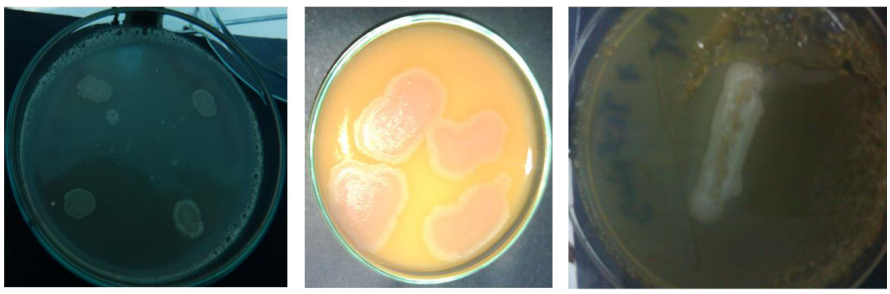
Additional test has been performed by studying the production of biosurfactants that emulsify hydrocarbons. Emulsion index (E24) was calculated as an indicator for Biosurfactants production. McFarland standards were used as a reference to adjust the turbidity of bacterial consortium [9]. The E24 was investigated by adding crude oil with LB medium in 1:1 ratio (v/v). The solution was vortexed for 5 min and incubated for 24 h. Emulsification percentage was calculated through the height of emulsion layer. In addition, E24 was determined for gasoline, diesel fuel or SAE 140. All the experiments were performed in triplicates. E24 = Height of emulsion layer/Total height of solution ×100.

3. Results

3.1. The Landscape of Enzymatic Activities of *L. louembei*

3.1.1. Lipolytic Activity

In order to show that *L. louembei* is able to secrete lipase enzymes in the extracellular medium, we used three agar media supplemented with olive oil, egg yolk and Tween 20. LB medium supplemented with olive oil, colonies appear smaller and surrounded by an opaque halo which testifies to the degradation of fatty acids (**Figure 1(a)**). The Mossel medium supplemented with egg yolk colonies are large pink in color surrounded by an opaque halo that testifies to lecithinase activity (**Figure 1(b)**). The LB medium supplemented with Tween 20 colonies grow from the depth of the streak to the surface with the formation of an opaque halo around the growth zone and with the supernatant the colony is very large occupying almost the middle of box (**Figure 1(c)**).



(a) (b) (c)

Figure 1. Lipolytic activity of *L. louembei on* different culture media. (a): on LB medium supplemented with olive oil; (b): on Mossel medium containing lecithin of egg yolk; (c): on LB medium supplemented with tween 20.

3.1.2. Proteolytic Activity

We have assessed the proteolytic activity at 37˚C by using casein substrate and we showed that *L. louembei* is able to secrete proteases in the extracellular environment. Figure 2 shows the result obtained from degradation of casein explaining the proteolytic activity of the supernatant of *L. louembei*. The experiment was performed four times for better reproducibility (Figure 2(a)). The clear zone around the wells indicates proteolytic digestion of the skimmed milk by proteases contained in the *L. louembei* supernatant (Figure 2(a)).

3.2. Effect of Temperature on Growth and Proteolytic Enzyme Activity

Extracellular proteolytic enzymes are usually constitutive and dependent on bacterial density. In order to correlate quorum sensing and proteolytic activity, we tested the enzymatic production of *L. louembei* at different incubation temperatures. The optical densities were taken in side event.

The results show that we cannot see any correlation between the growth and

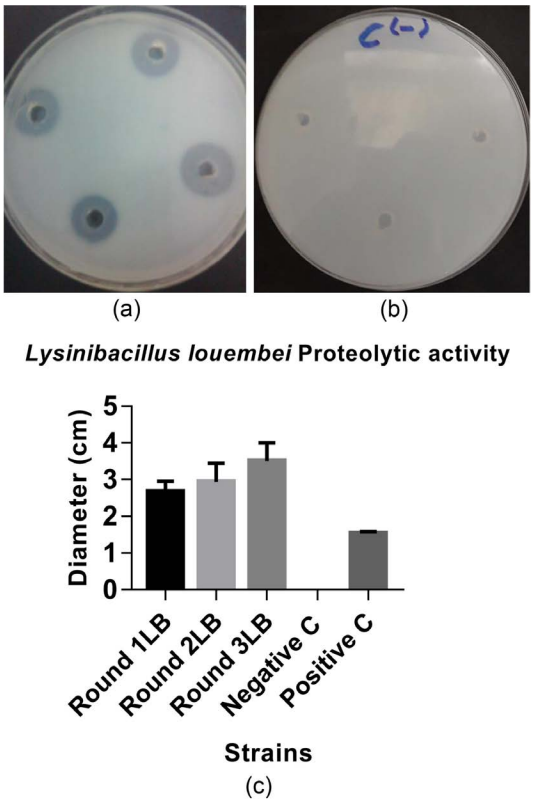


Figure 2. Proteolytic activity of *L. louembei* in milk agarose medium. (a) Supernatant of *Lysinibacillus louembei* deposited four times. (b) Supernatant of *Bacillus sp*, used as a negative control, (c) percentage of proteolytic activity by using *L. louembei*, Round1LB: the first round of *L. louembei Bacillus sp* used as negative control and *Bacillus Licheniformis* NM76 use as positive control.

the enzymatic activity because basing on 30˚C and 37˚C the activity is low by compare with 40˚C, 45˚C and 50˚C where the activities are very high (Figure 3).

The proteolytic activity being very interesting at 40˚C, 45˚C and 50˚C, we sought to know the stability of the protease at different temperatures. After incubation at 30˚C, 37˚C, 40˚C, 45˚C, 50˚C and 60˚C for $T_0 = 0$, $T_1 = 1$ h, $T_2 = 3$ h and $T_4 = 5$ h, 50 µL of supernatant were incubated at the afore mentioned temperature. The diameters were measured and results showed the protease secreted by *L. louembei* seems to be stable at these temperatures after the incubation time at 40˚C, 45˚C, and 50˚C (Figure 4).

3.3. Effect of pH on Growth and Proteolytic Enzyme Production

In the medium containing the LB, different pHs were adjusted. The growing was performed at 40˚C. Topic illustrates that diameters corresponding to the proteolytic activity varies between 2 to 3 cm at pH 7, 8 and 9 with the optimum at

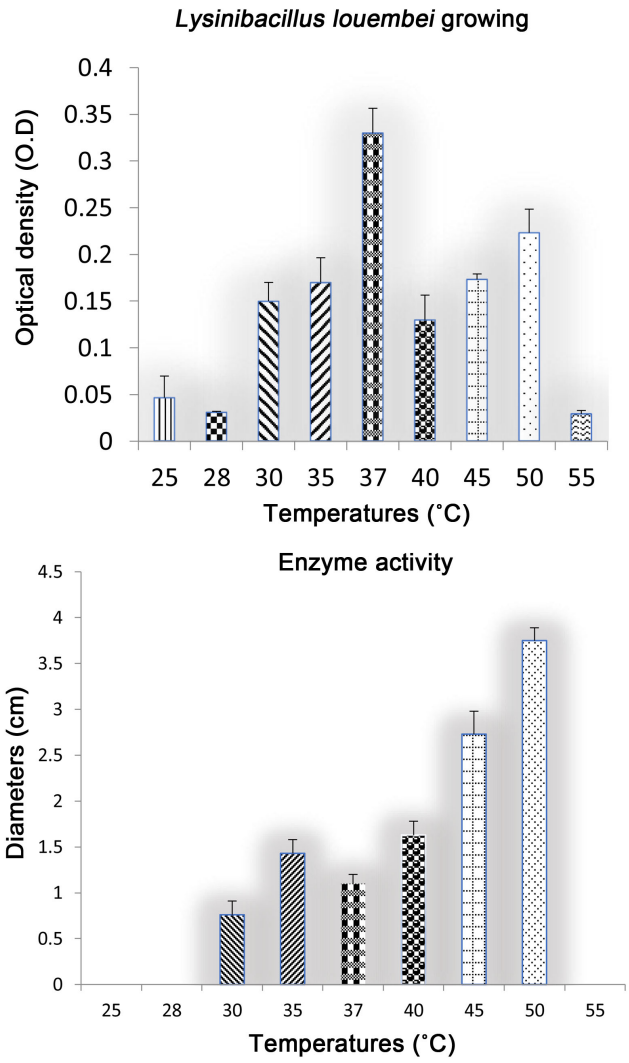


Figure 3. Variation of *L. louembeii* growing and caseinolytic activity with temperature.

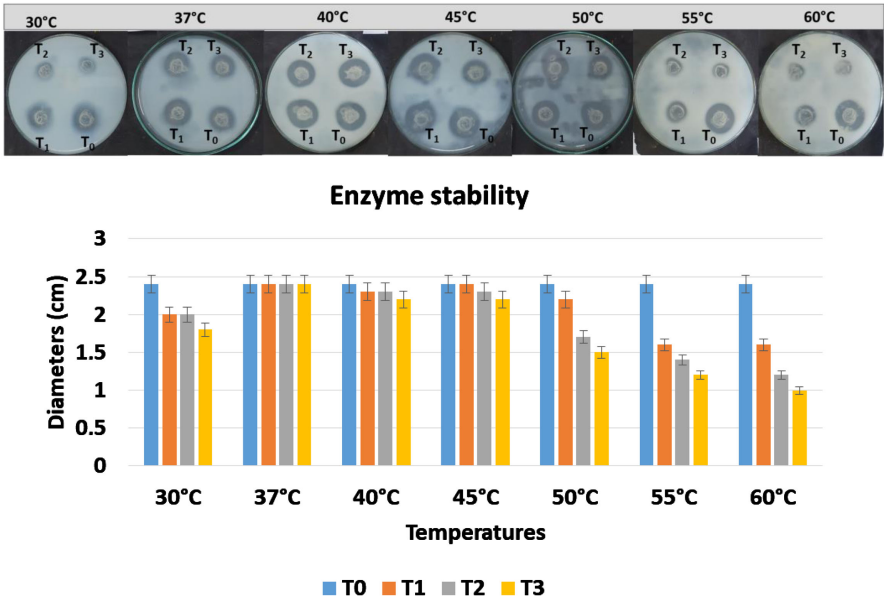


Figure 4. Caseinolytic protease stability. T_0: before incubation, T_1: after 1 h of incubation, T_2: after 3 h of incubation and T_3: after 5 h of incubation.

pH 8 (**Figure 5**). The activity is interesting by compare at pH 10.

3.4. Assessment of Amylolytic, Cellulolytic and Pectinolytic Activities

Amylolytic, Cellulolytic and Pectinolytic activities were carried out at different temperatures in order to verify the optimum of enzymatic production. The diameter of the halo was measured. The enzymatic activity was expressed in percent (% Ac = Td-Tw/Td, with %Ac which is the percentage of activity, Td the total diameter and Tw the diameter of the colony). Enzymes exhibit different activities depending on temperature (**Figure 6**). Cellulolytic and pectinolytic activities are very interesting at this incubation temperature range. All enzymes have activities ranging between 30˚C and 81% with a variation at 30˚C.

3.5. The Ability of *Lysinibacillus louembei* to Degrade and/or to Tolerate Hydrocarbons

The evaluation of the tolerance and/or the degradation of hydrocarbons in BH medium supplemented with hydrocarbons including SAE 140, gasoline or diesel fuel by the strain of *L. louembei*, have been tested in solid medium and in liquid one. The figure shows that *Lysinibacillus louembei* is able to grow in solid medium just after 24 h and in liquid as well (Data not shown). The Petri dishes was full of bacterial mass after 72 hours (**Figure 7**). Gram staining, catalase, oxidase, mannitol, growth temperature, optimum pH of culture, percentage in NaCl and 16S rRNA were used to confirm the strain.

To emphasize if *L. louembei* could involve in the production of biosurfactants with more successful, a qualitative test called emulsion index (E24) has been conducted by inoculating precultures in flasks containing the nutrient broth.

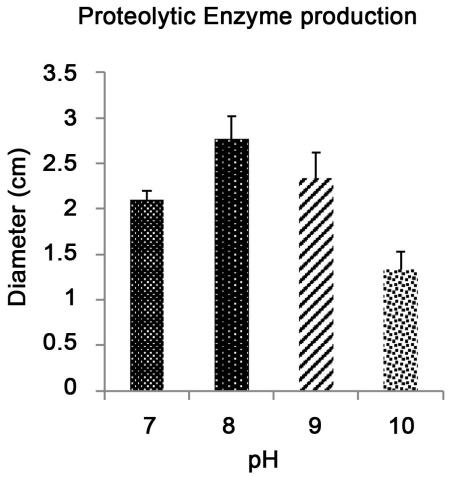


Figure 5. Influence of pH on growth and Production of the proteolytic enzyme.

Incubation has been done overnight at 37˚C. As results *L. louembei* is able to secrete biosurfactants by mixing with either gasoline or SAE140 which is engine oil. **Figure 8(a)** and **Figure 8(b)** illustrates the emulsification activity in the presence of both types of hydrocarbon. *E. coli* TOP10 and BL21 have been used as a negative control. The emulsion index is above to 50% in both cases. The average emulsion index for gasoline is 62.2% ± 2.62% and that for engine oil SAE 140 the average emulsion index is 85.97% ± 6.01% (**Figure 8(c)**).

4. Discussion

In this none previously reported study, we showed that *L. louembei* is a real enzyme manufacturing machine by harboring several extracellular enzyme activities including lipase, cellulase, amylase, protease and pectinase.

L. louembei therefore has proteolytic activity and may also have fibrinolytic activity. Any strain that has a caseinolytic activity also has fibrinolytic activity but the reverse is difficult [6].

In very recent study, *L. macrolides* and *L. fusiformis* isolated of from the gut of the subterranean termite *Psammotermes hypostoma* Desneux are able to digest cellulose [10] and xylan [11]. *L. louembei* has been isolated from Ntoba Mbodi, an alkaline fermented leaves of cassava from the Republic of the Congo [4]. Enzymatic landscapes could clearly explain the softening of leaves in three days by combining cellulolytic, amylolytic and pectinolytic activities. Those should be play a very important role in disrupting subtrats like cellulose, amylose and pectin.

Our findings have shown that the proteolytic activity is not correlated with the biomass. At 37˚C, the microbial biomass is very important, with low enzymatic activity. At 40˚C, the biomass is very low compared to 37˚C, while the enzymatic activity is important at this temperature. At 50˚C, the microbial growth is greater than that observed at 45˚C. With a very high enzymatic activity whereas at

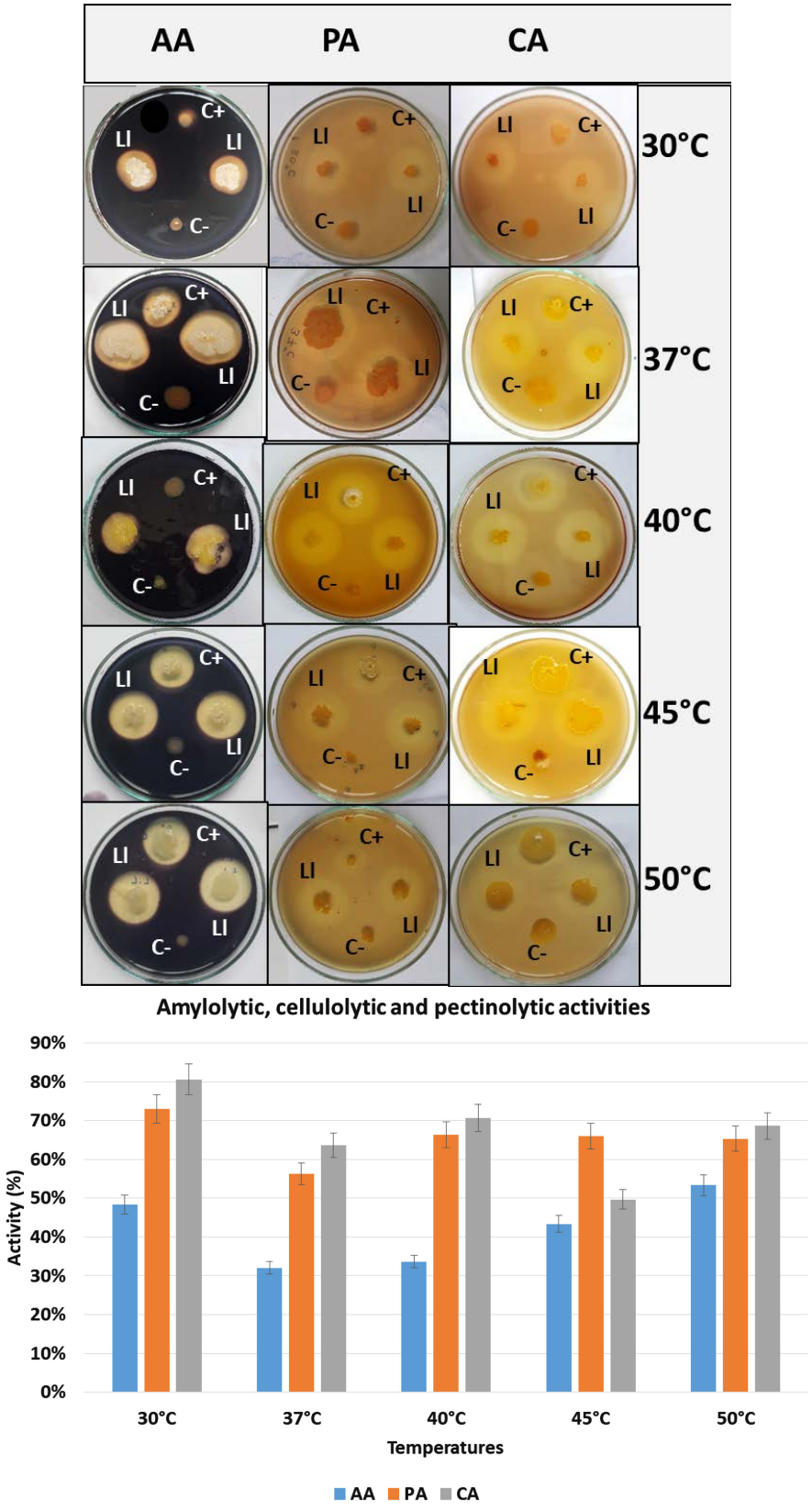


Figure 6. Amylolytic, cellulolytic and pectinolytic activities. C+: Positive Control; C–: negative control, Ll: *Lysinibacillus louembei*. AA: Amylolytic Activity, PA: Pectinolytic Activity, CA: Cellulolytic Activity.

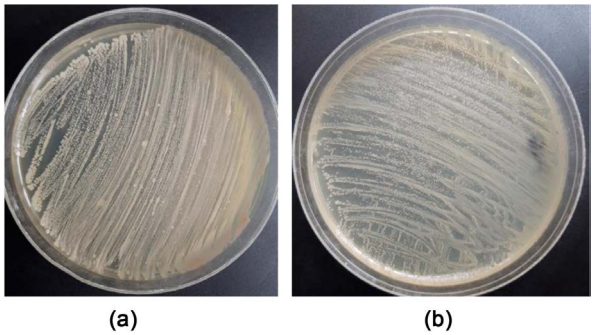


Figure 7. The Ability of *L. louembei* to grow in BH medium. (a): the BH has been supplemented with Gasoline (a). (b): the BH has been supplemented with diesel fuel (b). The growing has been stop after 72 h.

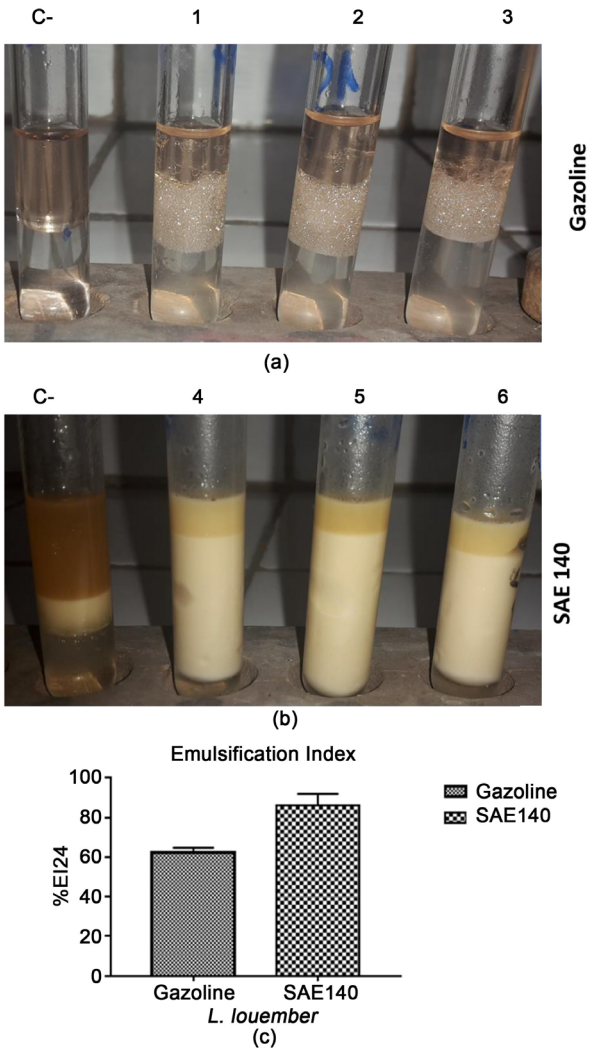


Figure 8. Production of Biosurfactant of *L. louembei*. (a): in the presence of gasoline, (b): in the presence of SAE140. (c): An overnight culture *E. coli* TOP10 used as negative control. 1, 2 and 3: experiment 1, 2 and 3 of gasoline. 3, 4 and 5: experiment 4, 5 and 6 of SAE140. (c): emulsification index.

55˚C, the bacterial growth is almost none with a lack of enzymatic activity as mentioned above. The results obtained showed an optimal activity optimum at about 45˚C - 50˚C.

The stability of the proteolytic enzyme is variable depending on the temperature and the incubation time. Indeed, in this work we have shown that this enzyme is thermostable in temperature range of 30˚C to 60˚C between 0 h and 5 h of incubation. In addition, this stability is interesting between 37˚C - 40˚C. Above 50˚C, this enzyme is still stable with a clear decrease in activity despite the fact that the bacteria no longer grow at these temperatures in contrary to studies previously demonstrated [12] [13]. Indeed, the *in vitro* analysis conditions do not necessarily reflect the physiological conditions *in vivo* and that environmental factors can positively or negatively influence the protein stability that can explain this result [14].

In this study, *L. louembei* shows the other activities (pectinolytic, cellulolytic and amylolitic) that can be influenced by temperature. The optimum of production was around 40˚C with activities varying between 66% and 71%, as shown in **Figure 6**, except amylolytic activity. The production is interesting at 30˚C with an activity of around 50% to 80% depending on the type of activity. There is less growth at this temperature. On the other hand, the amylolytic activity increases proportionally with the temperature of 37˚C to 50˚C. This enzyme may be of interest because enzymes with high temperature and thermodynamic stability are preferred for industrial applications [15]. Thus, purification of this enzyme which seems thermostable would be important in biotechnology. Amylases have been reported to have potential application in food fermentation, textile, paper and pharmaceutical industries [16]. These enzymes are most produced by microorganisms such as bacteria and yeasts [17]. Amylases represent nearly 30% of the global market in terms of enzyme production [18].

Other enzymes like cellulases have been shown to be interesting, are the cellulolytic thermostable activity [19]. We have shown that cellulase has the best activity at 30, 40 and especially 50˚C. These results show that the cellulase produced by *L. louembei* could be thermostable as previously reported even up to 60˚C [20]. It should be notified that thermostable cellulolytic enzymes have great potential in industrial processes such as the food industry, the textile industry and bioconversion [19] [20]. *L. louembei* can be used in cost-efficient cellulase production for bioconversion of a million residual biomass.

In this work, we have shown *L. louembei* has also the ability to tolerate and/or degrade hydrocarbons when grown in BH medium enriched in gas oil and SAE 140. Indeed, the growth of a bacterium requires, in addition to energy and a chain of electron carriers, a carbon source [21]. The BH medium used during this work is a mineral medium, the carbon source used by *L. louembei* therefore comes only from diesel and SAE 140 added to the culture medium. The results obtained at the end of our experiment indicate that, *L. louembei* uses as a carbon source the hydrocarbons tested. This comes to say that our strain is

able to tolerate and/or degrade the diesel and SAE 140. Many things are known about the fact that bacteria are able to grow on hydrocarbon-enriched BH medium, but there are few studies about enzymes that are secreted for the use of heavy and light hydrocarbons such as gasoline and gas oil. This study allows to postulate that *L. louembei* could secrete in its extracellular medium some oxydo-reductases, dehydrogenases and hydrolases for a good use of the carbon and the good functioning of the respiratory chain.

Furthermore it was also demonstrated that *L. sphaericus* DMT-7 isolated from diesel fuel contaminated soil can desulfurize dibenzothiophene (DBT) [22]. *L. sphaericus* plays an important role in bioremediation by degrading hydrocarbons in polluted areas and using them as the sole source of carbon [23]. Our findings are particularly interesting in terms of the ability to emulsify hydrocarbons with better hydrocarbon emulsification index that are around 60%. These observations thus confirm that *L. louembei* is capable of bioremediation by producing biosurfancts. In addition, A mesophilic strains *Lysinibacillus* sp has been isolated and showed the maximum crude oil degradation potentiality and biosurfactant has been partially purified and characterized by showing interest stability against temperature and salinity increasing and important emulsifying activity against oils and hydrocarbons [24]. Different type of Biosurfactant have been documented. This is included Rhamnolipid produced from a *L. sphaericus* IITR51 [25], 10,12-dihydroxystearic acid from *L. fusiformis* [26], glycolipid from *L. fusiformis* S9 [5] and non-identified biosurfactant from *Lysinibacillus* sp [27]. Proposals have been done for these microbial biosurfactants to treat cancer [28]. *Lysinibacillus louembei* could be use in bioremediation to detoxify or remove pollutants. This microorganism is very interesting for hydrocarbon bioremediation.

We showed that *Lysinibacillus* is able to solubilize gasoline, diesel fuel and SAE140. Recently it has been shown that *Lysinibacillus* spp can naturally solubilization and degrade polychlorinated biphenyls (PCBs) [29].

5. Conclusion

This study aimed to characterize the biomolecules secreted by *L. louembei* in the extracellular environment. The results showed that the cellular extract of *L. louembei* contains several enzymes including lipases, proteases, cellulases, pectinases and amylases which are capable of hydrolyzing polymers such as lipids, proteins, cellulose, pectin and amylose and/or amylopectin. This work has shown the enzymatic potential at an optimum between 40˚C and 50˚C at pH 8. In addition, we have shown that *L. louembei* is able to tolerate and/or to degrade light and heavy hydrocarbons, indicating the secretion of enzymatic landscape such as oxydo-reductases and hydrolases in the extracellular medium. This bacterium is also capable of emulsifying hydrocarbons with an emulsification index approaching 70%, proof of the production of biosurfactants in the extracellular environment. As part of the continuation of this work, mass spectrometry cha-

racterization tests (MALDI TOF) will be interesting to carry out for molecular identification of extracellular enzymes. An in-depth study of supernatant purification techniques may be feasible to show the chemical structure of biosurfactants by using HPLC techniques. Finally more attractive and exciting studies will be around the sequencing of *L. louembei* genome and this will open ways for recognizing these bacteria as safe for pharmaceutical and industrial applications.

Data Availability

The Excel sheet including the data used to support the findings of this study is available from the corresponding author upon request.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any intellectual commercial or financial relationships that could be construed as potential conflicts of interest.

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