Frontiers in Environmental Microbiology
2024; Vol. 10, No. 2, pp. 29-44
https://doi.org/10.11648/j.fem.20241002.11

Research Article

Isolation and Characterization of Indigenous Bacteria with Purifying Potential in Solid Palm Oil Extraction Sludge Generated by SOCAPALM-Mbambou

Tassong Saah Denis[1], Simo Claude[1, 2, *], Taffouo Victor Désiré[1]

[1]Laboratory of Plant Biology, Department of Plant Biology, Faculty of Science, University of Douala, Douala, Cameroon

[2]Laboratory of Plant Physiology, Department of Biological Sciences, Higher Teacher's Training College, University of Yaoundé I, Yaounde, Cameroon

Abstract

The oil palm industry contributes significantly to the economic development of producing countries such as Cameroon. Unfortunately, the exploitation of palm oil constitutes a source of environmental pollution due to the production of enormous quantities of waste during its extraction process, including solid sludge generating greenhouse gases which contribute to global warming climatic. All this leads to the search for alternatives which consists of isolating and characterizing indigenous bacteria with biodegradation capacities in sludge from palm oil extraction. The pH and bacterial counts were determined by the potentiometric method and the decimal dilution technique, respectively. The isolated bacteria were identified by their cultural, cellular and biochemical characteristics. In addition, the identification of Gram- bacteria was further explored by the API 20 E gallery. The palm oil biodegradability test was carried out on M2 medium supplemented with 2% palm oil. The solid sludge biodegradability test was carried out on liquid MSM medium supplemented with 4% sludge stock solution. The results showed that the sludge sample had a slightly alkaline pH of 7.3. A bacterial load of around 10^9 CFU/g of soil was counted. Thirty-one bacterial strains were isolated and purified, including 12 *Bacillus sp*, 10 *Pseudomonas sp*, 8 *Proteus mirabilis* and 1 *Klebsiella pneumoniae*. All isolates tested for their ability to degrade palm oil or solid sludge grew in culture media with palm oil or solid sludge as the sole source of carbon and energy but with a difference in load. Thus, isolates BI2, BI5, BI31, BI10 and BI 9 showed the highest degradation capacities. These isolates could be used to constitute consortia of microorganisms that can be used in the treatment of waste generated by palm oil extraction.

Keywords

Biodegradation, Consortium, Isolate, Microorganism, Solid Sludge

1. Introduction

The oil palm (*Elaeis guineensis*) is a perennial monocot plant of the *Arecaceae* family, native to West Africa which is mainly cultivated for its palm oil extracted from the pericarp of the fruit [1]. Palm oil plays a key role in meeting global food needs and contributes significantly to the economic development of producing countries. In Cameroon, a production of 465,000 t was

*Corresponding author: simoclaude@yahoo.fr (Simo Claude), simoclaude236@gmail.com (Simo Claude)

Received: 7 March 2024; **Accepted:** 25 March 2024; **Published:** 17 April 2024

recorded during the 2021-2022 harvest campaign, placing it third in Africa and twelfth in the world behind Nigeria (1,400,000 t) and Ivory Coast (600,000 t) [2]. The multiplication of uses of palm oil (cosmetics, energy, biofuels, agri-food, etc.) has increased its demand. Currently, overall demand exceeds supply, and this trend is likely to strengthen in the future, making it a particularly attractive product for investors. According to forecasts from the OECD and the FAO, global palm oil production is expected to increase by 20% between 2021 and 2030. Supply therefore still remains largely in deficit compared to strong demand [3]. According to FAO, 21 million hectares of forest in the world in general and 170,169 ha in Cameroon have been destroyed in favor of palm oil plantations [4]. As a result, forest clearing not only destroys biodiversity, but also endangers existing microflora and fauna. In addition, the slow decomposition and fires of peatlands, resulting from the expansion of oil palm plantations, have caused a significant loss of biodiversity as well as high greenhouse gas emissions [5]. The cultivation policy implemented by oil growers in Cameroon as in all producing countries aims to increase production yield in order to satisfy high demand, which leads them to use enormous quantities of chemical fertilizers in their practices fertilization. According to [6], the application of nitrogen fertilizers is the primary source of greenhouse gas emissions in plantations. In addition, some of these fertilizers are responsible for the pollution of surface water and groundwater. Hence the need to find non-polluting alternatives in fertilization practices. In this context, replacing mineral fertilizers with organic fertilizers, such as compost, could provide a solution to this problem [7]. Although the oil palm industry is recognized for its contribution to economic growth and rapid development in Cameroon, it also contributes to environmental pollution due to the production of huge quantities of waste during the process extraction. These wastes are mainly liquid discharges forming a final effluent called POME (Palm Oil Mill Effluent), stalks or EFB (Empty Fruit Bunch), solid sludge from three-phase centrifugation, fibers and hulls [8]. Managing this waste poses enormous problems for agro-industries. Some of this waste, such as fibers and hulls, is regularly used for the production of energy in boilers. In most industries, liquid effluents are treated in a lagoon system before discharge into waterways, but this treatment is not very effective due to the high content of the clarification sludge which quickly loads into the water. the settling basin and contaminate the other basins, hence the high levels of biochemical oxygen demand (BOD), chemical oxygen demand (COD) and suspended matter (SM). Therefore, it is necessary to regularly clean the sludge from the settling basins and treat it otherwise. The method of managing stalks as well as solid sludge practiced by agro-industries in Cameroon is their dumping in the field for their natural composting. However, this practice causes environmental pollution and deteriorates the surrounding environment. Solid sludge and cobs take several years to degrade naturally when dumped in the field. In contact with plants, palm oil sludge inhibits their growth and causes their death [8]. In addition, the leaching of sludge by rainwater causes water depletion and leads to aquatic pollution. Furthermore, solid sludge is responsible for bad odors and therefore a source of diseases. Given the abundance of this sludge generated by palm oil agro-industries in Cameroon and their impact on environmental pollution, there is an urgent need to put in place an effective management system for their treatment, in order to preserve the environment. With this in mind, biological treatment by composting can be a good option for the sustainable management of this waste. Composting would make it possible to treat this waste rich in organic matter and the opportunity to use it in the field as organic fertilizer. This treatment system would significantly reduce the use of chemical fertilizers and the environmental impact of oil palm plantations. Indeed, this method relies on the ability of bacteria to use fatty acids as a source of carbon in their metabolism. The method requires competent bacteria to be available for its implementation. To do this, the principle is to isolate this type of bacteria in soils polluted by fatty acids. However, Cameroonian companies operating in the field of industrial waste treatment, particularly biological treatment, always use laboratories in Western or Asian countries to obtain microorganisms suitable for biodegradation, which is very expensive and therefore makes the cost very high waste processing for local businesses. However, starting from the hypothesis according to which, for each type of waste accumulated in the environment, certain microorganisms can adapt to live there and use it as a source of carbon and therefore can degrade this waste, it is therefore possible to isolate these indigenous microorganisms and use them in the biodegradation process. These microorganisms could be used to constitute consortia of microorganisms that can be used in the treatment of waste generated by palm oil extraction.

2. Materials and Methods

2.1. Materials

The palm oil sludge samples used in this study were taken from the Cameroonian palm grove company (SOCAPALM-Mbambou) site located in Dizangue-Cameroun in the Littoral region, Sanaga maritime Division, Dizangue Sub-division.. The palm oil used as substrate was purchased on the local market. This oil was then sterilized in an autoclave before being used.

2.2. Methods

2.2.1. Evaluation of Hydrogen Potential

The pH of the sample was measured using a "SARTORIUS AGCOTTINGEN" type pH meter. A suspension of solid sludge was prepared in distilled water at 5/50 (mass/volume ratio). Stirring was carried out for a period of one hour followed by decantation, then the probe was immersed in the solution and the value was recorded.

2.2.2. Isolation, Purification and Storage of Bacterial Isolates

The serial decimal dilution technique was used to enumerate total mesophilic aerobic microflora in sludge samples [9, 10]. The dilution series ranging from 10^{-1} (stock solution) to 10^{-8} was prepared from a suspension of 10g of sludge in 90 mL of sterile distilled water. Subsequently, 0.1 mL of each dilution was plated in two repetitions on the Petri dishes containing ordinary nutrient agar (NA). The boxes were incubated at 37 ℃ for 24 hours. At the end of the incubation, the colonies presenting morphological differences were counted in CFU (Colony Forming Unit) and the number of germs per gram of the sample was determined according to the formula of Marchal and Bourdon (1982) [11]:

$$N = n / d.v$$

where N: number of microorganisms in CFU/mL; n: number of colonies counted; v: Volume collected (0.1mL); d: Dilution.

The colonies counted were purified by successive and alternating subcultures in liquid medium, then in solid medium [12]. After purification, the isolates were stored in Eppendorf tubes at 4 ℃ on solid medium (NA) and at 20 ℃ in nutrient broth supplemented with glycerol (1:1, v/v) [13].

2.2.3. Cultural and Cellular Characterization of Isolates

(i). Macroscopic Appearance

Observing the macroscopic appearance of the colonies allows an initial characterization to be carried out. According to Joffin and Leyral (2006), the macroscopic identification elements are as follows: shape, color, elevation, contours, size, opacity, consistency and surface [14]. Mossel medium supplemented with polymixin B (1 mg/mL), a medium specific to the growth of *Bicillus*, was used to verify the capacity of bacteria suspected to belong to the genus *Bacillus sp.*, according to the modified method of Goma-Tchimbakala *et al.* (2020) [10].

(ii). Microscopic Appearance

An observation under a microscope of the bacteria in the fresh state made it possible to see the mobility. The shape and pattern of grouping were determined by methylene blue staining. A Gram stain was done to classify the bacteria according to bacterial type and confirm their purity in addition to Malachite green staining to see the presence of resistance spores [15].

2.2.4. Biochemical Characterization of Isolates

Orientation Test: Potash Test (3% Aqueous KOH Solution)

The potash test was carried out in addition to the classic Gram staining method. The KOH test was carried out according to the method described by Carlone *et al.* (1983) taken up by Selmoun *et al.* (2016) [16, 15]. On a glass slide, a drop of 3% potassium hydroxide was pipetted and then a visible loop of cells from a single, well-isolated colony was mixed into the drop. When the mixture becomes viscous and stringy within 60 seconds after mixing (KOH positive), the colony is considered Gram negative.

(a). Characterization of Gram+ Bacteria

Search for oxidase

The oxidase test was carried out according to the method of [17]. An oxidase disk previously soaked with a drop of sterile distilled water was placed on a slide and brought into contact with a freshly cultured bacterial colony. The appearance of a purple color directly obtained indicates that the test is positive.

Search for catalase

Using a platinum loop, a drop of the bacterial suspension is placed on a glass slide then emulsified in a drop of 10 volume hydrogen peroxide. The presence of catalase is immediately manifested by gas bubbles (effervescence) corresponding to the released oxygen [11].

(b). Characterization of Gram- bacteria

In addition to the search for respiratory enzymes such as catalase and oxidase, the other biochemical characteristics of the Gram- bacterial isolates were determined using an API 20E identification gallery (BioMérieux). Seeding of the galleries was carried out from 24-hour colonies following the protocol proposed by the manufacturer. The galleries were incubated at 37 ℃ for 48 h. The reactions produced during incubation are reflected by the change in the colored indicator. The results were noted on the result sheet provided by the manufacturer and the identification was made using the APIWEB identification program accessible online.

2.2.5. Biodegradability Test of Palm Oil and Extraction Sludge Using Isolates

(i). Palm oil Biodegradability Test

M2 medium supplemented with 2% palm oil was used for this test. Two agar plates were provided for each strain: one without yeast extract and the other with 1g/L of yeast extract in order to make a comparison and confirm the degradation capacity of palm oil as the sole source of carbon and energy by these bacteria. This test was followed for five days. The degradation efficiency of the isolates was determined by comparing the growth speed and colony load [15].

(ii). Biodegradability Test of Solid Sludge from Palm Oil Extraction

This test was carried out by monitoring the growth kinetics of the bacteria according to the method of [18]. Thus 10g of solid sludge was dissolved in 1L of sterile distilled water to form the stock solution. The pre-cultures of the 8 bacterial isolates were carried out according to the protocol

of Temesgen *et al.* (2018) whose composition of the nutrient broth in grams per liter was as follows: Glucose: 3g/L; NH_4CL: 3.2 g/L; KH_2PO_4-: 1 g/L; K_2HPO_4-: 10 g/L; $MgSO_4.7H_2O$: 1g/L; NaCL: 5 g/L; peptone: 5 g/L [19]. Each 24-h pure isolate collected from the nutrient agar was inoculated into a vial containing 50mL of sterile nutrient broth and the set of vials was incubated on a shaker at 32 ℃ for 48h. The liquid MSM medium supplemented with 4% stock solution of palm oil extraction sludge (v/v) used for the test has a salt composition similar to that of the pre-culture nutrient broth but free of glucose and peptone. For each series of tests, the pre-cultures of the isolates were centrifuged at 4000 rpm for 10 minutes, the resulting pellets were washed with 0.9% NaCL solution and centrifuged once more. The recovered pellets were each suspended in 20 mL of sterile 0.9% NaCL solution and calibrated with a spectrophotometer at 600 nm at OD=0.1 corresponding to a load of 107 CFU/mL. In each series of tests, 5mL of calibrated cell suspension of each isolate was inoculated in triplicate into the 100mL vials containing 45mL of sterile supplemented MSM solution. A vial containing sterile, non-inoculated supplemented liquid MSM medium into which 5 mL of 0.9% NaCL solution was introduced served as a control. OD measurements were performed after every 24 h for 7 days of incubation in a shaker at 30 ℃ starting on day 0. OD measurements were used to determine the growth rate of each isolate after 5 days incubation according to the formula:

$$T\ (\%) = [(ODf-ODi)/ODi] \times 100$$

where ODf: final optical density; ODi: initial optical density.

2.2.6. Data Analyzes

The data collected were subjected to an analysis of variance (ANOVA) with the aim of investigating the existence of a significant difference between the isolates. The separation of isolates into homogeneous groups was carried out by the Duncan test at the α=5% threshold. The dendogram of morphological characters made it possible to group isolates with similar characters. All of these analyzes were carried out using SPSS 20.0 software.

3. Results

3.1. Evaluation of Hydrogen Potential

The sludge sample studied for microbiological analysis has a slightly alkaline pH of 7.3 after pH meter stability which lasted nearly 30 minutes.

3.2. Isolation, Purification and Storage of Bacterial Isolates

The counts on nutrient agar obtained for the different dilutions after 48 hours of incubation provided an idea of the bacterial load of the sample. Observation of the sample shows a fairly high bacterial load around $12.6.10^9$ CFU/g of sludge. Based on all the cultural characteristics, 31 bacteria were isolated and purified from the solid sludge sample.

3.3. Cultural and Cellular Characterization of Bacterial Isolates

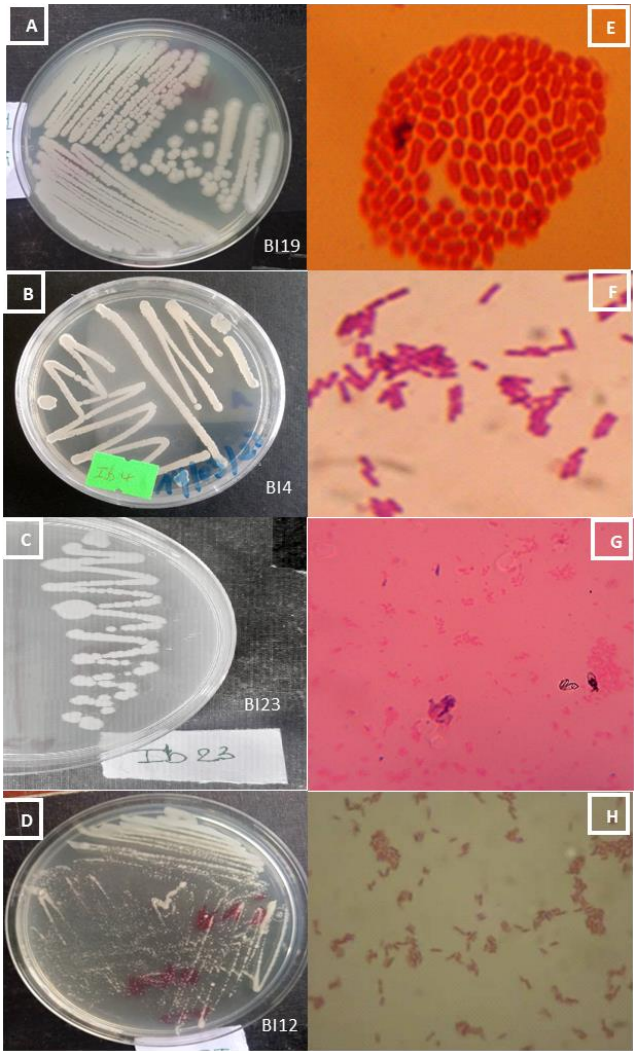

Figure 1. *Cultural (A, B, C, D) and cellular (E, F, G, H) (Gx100) aspects of the respective isolates BI19, BI4, BI23 and BI12.*

All the macroscopic and microscopic characteristics studied on the 31 isolates are presented in the following two Tables 1 and 2. According to these results, we note the presence of significant bacterial diversity in the sludge sample. Crop characteristics vary from one isolate to another. Bacterial isolates BI1, BI2, BI3, BI4, BI8, BI14, BI15, BI16, BI18, BI19, BI21 and BI26 all have large, round, flat, rough, opaque, dry, beige-colored colonies (except Ib16 which is whitish) and develop on Mossel medium. Their cells are Gram+,

rod-shaped, isolated, mobile and produce spores. Bacterial isolates BI5, BI6, BI7, BI9, BI10, BI11, BI12, BI13, BI20, BI22, BI23, BI24, BI25, BI27, BI28, BI29, BI30, BI31 all have round or irregular, beige-colored, invasive colonies, rounded, smooth and translucent. Figure 1 below presents the cultural and cellular aspects of some isolates. Their cells are Gram-, rod-shaped, isolated, mobile (with the exception of BI17 which is immobile). Figure 1 below presents the cultural and cellular aspects of some isolates. These cultural and cellular characteristics allow us to have a first idea of the type of bacteria. All 31 isolates are bacilli, including 12 Gram+ bacilli and 19 Gram- bacilli.

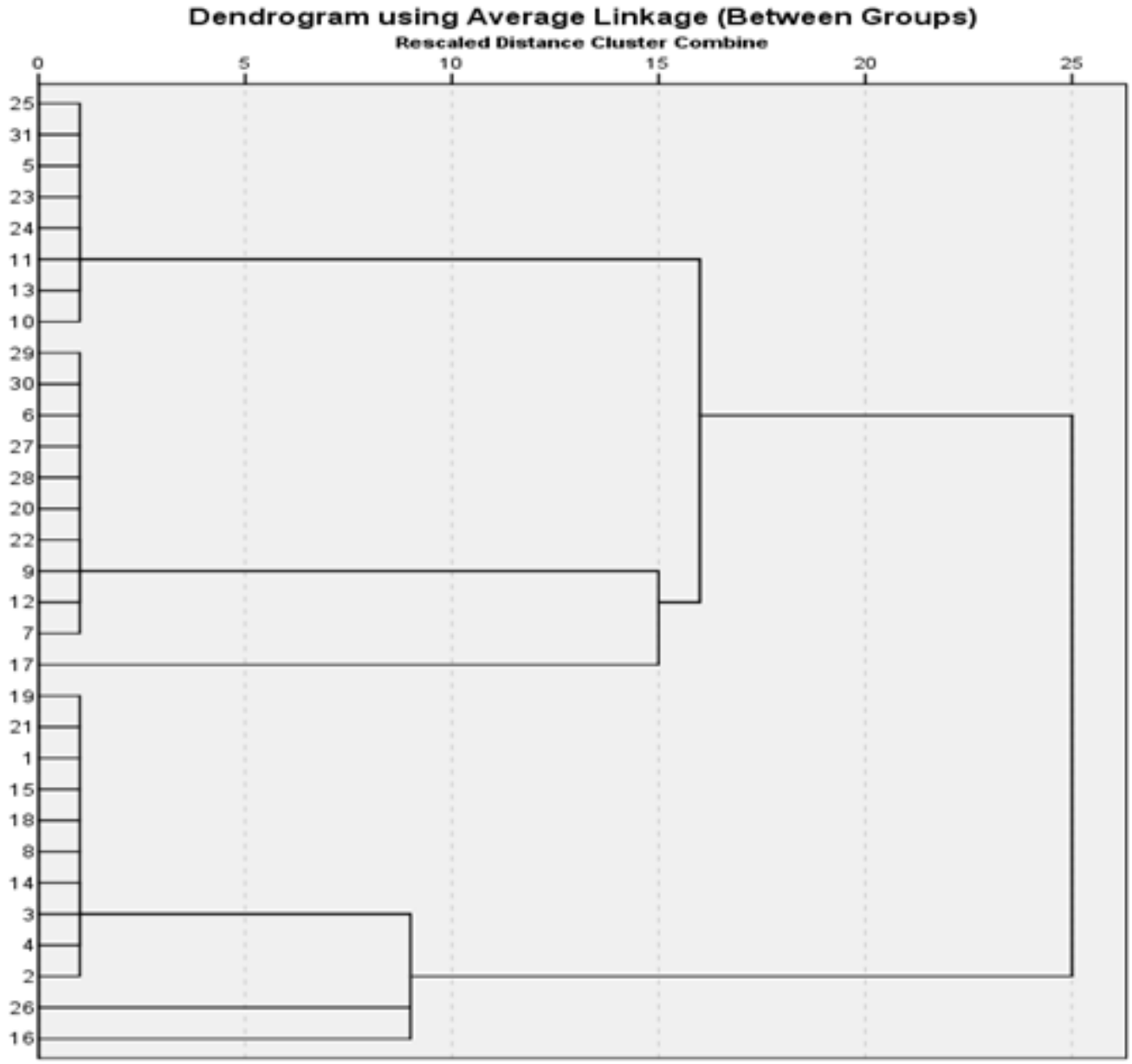

Figure 2. *Hierarchical classification of bacterial isolates obtained on the basis of cultural characteristics.*

The cultural characteristics of the isolates made it possible to classify them hierarchically. The various groups of bacteria were carried out at the threshold of 95% homogeneity (Figure 2). This hierarchical classification made it possible to distinguish two large groups of bacteria. Group 1 made up of 19 bacterial isolates (BI5, BI6, BI7, Ib9, BI10, BI11, BI12, BI13, BI17, BI20, BI22, BI23, BI24, BI25, BI27, BI28, BI29, BI30 and BI31) which all have the Round or irregular colonies, beige in color, invasive, rounded, smooth and translucent. Group 2 made up of 12 bacterial isolates (BI1, BI2, BI3, BI4, BI8, BI14, BI15, BI16, BI18, BI19, BI21 and Ib26) which all have large colonies, round, flat, rough, opaque, dry, of beige color (with the exception of Ib16 which is whitish) and develop on Mossel medium. Group 1 presents three subgroups: subgroup 1 consisting of bacterial isolate BI5, BI10, BI11, BI13, BI23, BI24, BI25 and BI31; subgroup 2 consisting of

bacterial isolates BI6, BI7, BI9, BI12, BI20, BI22, BI27, BI28, BI29 and BI30 and subgroup 3 consisting of bacterial isolate BI17. Group 2 also presents 3 subgroups: subgroup 1 consisting of bacterial isolates BI1, BI2, BI3, BI4, BI8, BI14, BI15, BI18, BI19, BI21; subgroup 2 consisting of bacterial isolate BI26 and subgroup 3 consisting of bacterial isolate BI16.

Table 1. *Cultural characteristics of bacterial isolates.*

Isolate code	Form	Color	Elevation	Outline	Size	Surface	Opacity	Consistency	Growth on Mossel medium
BI1	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI2	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI3	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI4	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI5	Rounds	Beige	Domed	NA	Invasive	Smooth	Translucent	NA	NA
BI6	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI7	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI8	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI9	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI10	Rounds	Beige	Domed	NA	Invasive	Smooth	Translucent	NA	NA
BI11	Rounds	Beige	Domed	NA	Invasive	Smooth	Translucent	NA	NA
BI12	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI13	Rounds	Beige	Domed	NA	Invasive	Smooth	Translucent	NA	NA
BI14	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI15	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI16	Rounds	Whitish	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI17	Rounds	Beige	Domed	Réguli ères	Small	Smooth	Translucent	Mucosa	NA
BI18	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI19	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI20	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI21	Rounds	Beige	Flat	Irregular	Large	Rough	Opaque	Dried	+
BI22	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI23	Rounds	Beige	Domed	NA	Invasives	Smooth	Translucent	NA	NA
BI24	Rounds	Beige	Domed	NA	Invasive	Smooth	Translucent	NA	NA
BI25	Rounds	Beige	Domed	NA	Invasive	Smooth	Translucent	NA	NA
BI26	Rounds	Beige	Domed	Irregular	Large	Rough	Opaque	Dried	+
BI27	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI28	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI29	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI30	Irregular	Beige	Domed	Irregular	Punctiform	Smooth	Translucent	NA	NA
BI31	Rounds	Beige	Domed	NA	Invasive	Smooth	Translucent	NA	NA

+: growth on Mossel medium plus polymixin B; NA: not applied.

Table 2. *Cellular characteristics of bacterial isolates.*

Isolate code	Gram stain	cell shape	arrangement	Mobility	sporulation
BI1	Gram +	stick	Isolated	Mobile	+
BI2	Gram +	stick	Isolated	Mobile	+
BI3	Gram +	stick	Isolated	Mobile	+
BI4	Gram +	stick	Isolated	Mobile	+
BI5	Gram -	stick	Isolated	Mobile	NA
BI6	Gram -	stick	Isolated	Mobile	NA
BI7	Gram -	stick	Isolated	Mobile	NA
BI8	Gram +	stick	Isolated	Mobile	+
BI9	Gram -	stick	Isolated	Mobile	NA
BI10	Gram-	stick	Isolated	Mobile	NA
BI11	Gram-	stick	Isolated	Mobile	NA
BI12	Gram-	stick	Isolated	Mobile	NA
BI13	Gram-	stick	Isolated	Mobile	NA
BI14	Gram +	stick	Isolated	Mobile	+
BI15	Gram +	stick	Isolated	Mobile	+
BI16	Gram +	stick	Isolated	Mobile	+
BI17	Gram-	stick	Isolated	Immobile	NA
BI18	Gram +	stick	Isolated	Mobile	+
BI19	Gram +	stick	Isolated	Mobile	+
BI20	Gram-	stick	Isolated	Mobile	NA
BI21	Gram +	stick	Isolated	Mobile	+
BI22	Gram -	stick	Isolated	Mobile	NA
BI23	Gram -	stick	Isolated	Mobile	NA
BI24	Gram -	stick	Isolated	Mobile	NA
BI25	Gram-	stick	Isolated	Mobile	NA
BI26	Gram+	stick	Isolated	mobile	+
BI27	Gram-	stick	Isolated	Mobile	NA
BI28	Gram -	stick	Isolated	Mobile	NA
BI29	Gram -	stick	Isolated	Mobile	NA
BI30	Gram-	stick	Isolated	Mobile	NA
BI31	Gram-	stick	Isolated	Mobile	NA

NA: not applied; +: positive sporulation.

3.4. Biochemical Characterization of Isolates

3.4.1. Characterization of Gram+ Bacteria

The search for respiratory enzymes such as oxidase and catalase made it possible to characterize the Gram+ bacteria isolated from the sludge sample. The reactions of the bacterial colonies deposited on an oxidase disk soaked in a drop of distilled water led to the immediate appearance of a purple color (positive test) in all the gram+ bacteria, indicating the

presence of phenylenediamine- oxidase or cytochrome oxidase in these bacteria.

The reactions of bacterial colonies emulsified in a drop of hydrogen peroxide on a glass slide led to the immediate appearance of gas bubbles (effervescence) corresponding to the oxygen released in all Gram+ bacteria, thus indicating the presence of catalase in the bacteria tested.

On the basis of the cultural (shape, color and size of colonies), cellular (color, shape, Gram staining, shape, arrangement, mobility, sporulation and growth on Mossel medium) and biochemical (catalase and oxidase) characteristics obtained, the isolates can be identified to the genus level. The bacterial isolates BI1, BI2, BI3, BI4, BI8, BI14, BI15, BI16, BI18, BI19, BI21 and BI26 all have large colonies, circular in shape, beige in color (with the exception of BI16 which is whitish) and develop in the Mossel environment. Their cells are Gram+, rod-shaped, isolated, mobile and produce spores. Biochemical tests indicate that all of these bacteria produce catalase (Table 3). All of these characteristics lead to their identification as belonging to the genus *Bacillus sp*.

Table 3. *Results of oxidase and catalase tests in gram+ bacteria.*

Stem code	Gram test	Catalase test	Oxidase test
BI1	Gram +	Positive	Positive
BI2	Gram +	Positive	Positive
BI3	Gram +	Positive	Positive
BI4	Gram +	Positive	Positive
BI5	Gram -	Positive	Negatif
BI6	Gram -	Positive	Positive
BI7	Gram -	Positive	Positive
BI8	Gram +	Positive	Positive
BI9	Gram -	Positive	Positive
BI10	Gram-	Positive	Negatif
BI11	Gram-	Positive	Negatif
BI12	Gram-	Positive	Positive
BI13	Gram-	Positive	Negatif
BI14	Gram +	Positive	Positive
BI15	Gram +	Positive	Positive
BI16	Gram +	Positive	Positive
BI17	Gram-	Positive	Negatif
BI18	Gram +	Positive	Positive
BI19	Gram +	Positive	Positive
BI20	Gram-	Positive	Positive
BI21	Gram +	Positive	Positive
BI22	Gram -	Positive	Positive
BI23	Gram -	Positive	Negatif
BI24	Gram -	Positive	Negatif
BI25	Gram-	Positive	Negatif
BI26	Gram +	Positive	Positive
BI27	Gram-	Positive	Positive
BI28	Gram -	Positive	Positive
BI29	Gram -	Positive	Positive
BI30	Gram-	Positive	Positive
BI31	Gram-	Positive	Negatif

3.4.2. Characterization of Gram- Bacteria

The results of the biochemical tests carried out on Gram- bacteria are grouped in Tables 4 and 5. On the basis of the cultural, cellular and biochemical characteristics obtained, certain strains can be identified at the genus or species level. Bacterial isolates BI5, BI6, BI7, BI9, BI10, BI11, BI12, BI13, BI20, BI22, BI23, BI24, BI25, BI27, BI28, BI29, BI30, BI31 all have round or irregular, beige-colored, invasive colonies, smooth and translucent. Their cells are Gram-, rod-shaped, isolated, mobile (with the exception of Ib17 which is immobile). Biochemical tests indicated that all of these bacteria produce catalase. In addition, the ONPG, CIT, GEL and OX tests are positive for bacterial isolates BI6, BI7, BI9, BI12, BI20, BI22, BI27, BI28, BI29 and BI30. These isolates are identified as belonging to the genus *Pseudomonas sp*. However, ODC (Ornithine decarboxylase), CIT (Citrate), IND (Indole), VP (Voges-Proskauer), GEL (Gelatin), GLU (Glucose), MAN (Mannose), INO (Inositol), SOR (Sorbitol), RHA (Rhamnose), BAG (Sucrose), MEL (Melibiosis), AMY (Amygdalin) and ARA (Arabinose) tests are positive for bacterial isolates BI5, BI10, BI11, BI13, BI23, BI24, BI25 and BI31. These isolates are identified as belonging to the species *Proteus mirabilis*. However, ONPG (O-nitrophényl-D-galactopyranoside), ADH (Arginine dihydrolase), LDC (Lysine decarboxylase), URE (Urea), CIT (Citrate), IND (Indole), VP (Voges-Proskauer), GEL (Gelatin), GLU (Glucose), MAN (Mannose), INO (Inositol), SOR (Sorbitol), RHA (Rhamnose), BAG (Sucrose), MEL (Melibiosis), AMY (Amygdalin) and ARA (Arabinose) are positive for bacterial isolate BI17. This bacterial isolate is identified as belonging to the species *Klebsiella pneumoniae*.

Table 4. *Results of the 11 biochemical tests of Gram- bacteria based on the API20E Kit and the oxidase test.*

	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	Species name
BI23	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI29	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI6	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI24	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI5	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI7	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI28	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI22	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI20	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI30	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI10	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI25	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI12	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI27	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*
BI13	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI31	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI17	+	+	+	-	+	-	+	-	-	+	-	*Klebsiella pneumoniae*
BI11	-	-	-	+	+	-	-	-	+	+	+	*Proteus mirabilis*
BI9	+	-	-	-	+	-	-	-	-	-	+	*Pseudomonas sp*

+: positive test; -: negative test.

Table 5. *Results of the 10 biochemical tests of Gram- bacteria based on the API20E Kit and the oxidase test.*

	GLU	MAN	INO	SOR	RHA	BAG	MEL	AMY	ARA	OX	Species name
BI23	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI29	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*
BI6	-	-	-	-	-	-	-	+	-	+	*Pseudomonas sp*
BI24	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI5	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI7	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*
BI28	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*
BI22	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*
BI20	-	-	-	-	-	-	-	+	-	+	*Pseudomonas sp*
BI30	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*
BI10	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI25	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI12	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*

	GLU	MAN	INO	SOR	RHA	BAG	MEL	AMY	ARA	OX	Species name
BI27	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*
BI13	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI31	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI17	+	+	+	+	+	+	+	+	+	-	*Klebsiella pneumoniae*
BI11	+	+	+	+	+	+	+	+	+	-	*Proteus mirabilis*
BI9	-	-	-	-	-	-	-	-	-	+	*Pseudomonas sp*

+: positive test; -: negative test.

3.5. Biodegradability Test of Palm Oil and Extraction Sludge Using Bacterial Isolates

3.5.1. Palm Oil Biodegradability Test

The assessment of palm oil biodegradability is based on the ability of isolates to use palm oil as the sole source of carbon and energy. Figure 3 shows some images of the growth of the isolates on M2 medium supplemented with 2% palm oil as the sole carbon source and on M2 medium supplemented with 2% palm oil plus yeast extract. It appears that all the bacterial isolates tested grew on the M2 medium supplemented with 2% palm oil and devoid of yeast extract after 48 hours with a difference in the load. Unlike, in boxes containing M2 medium with 2% palm oil and 1g/L of yeast extract, growth is obtained after 24 hours with a heavier load. Table 6 presents the results of the biodegradability test of palm oil by the different bacterial isolates. We observe that all the isolates grew in the presence of palm oil as the sole source of carbon and energy but with a difference in the load. Bacterial isolates BI2, BI5, BI9, BI10, BI13, BI22, BI29 and BI31 showed growth with higher load. These isolates were selected for further work.

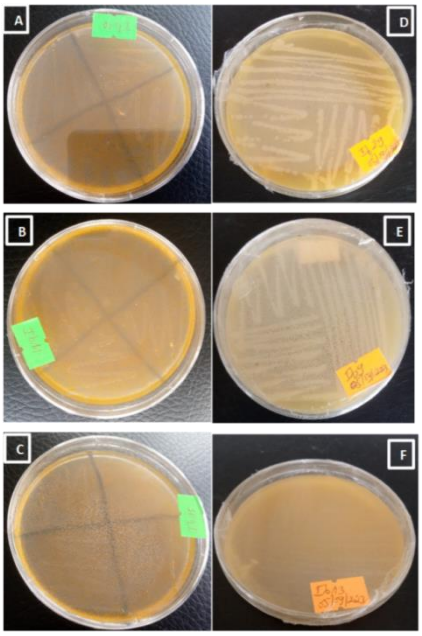

Figure 3. *Some images of the growth of bacterial isolates on M2 medium with 2% palm oil as the sole carbon source (A, B, C) and on M2 medium supplemented with palm oil plus yeast extract (D, E, F).*

Table 6. *Test de biodégradabilité de l'huile de palme par les différents isolats bactériens.*

Bacterial isolates	Growth in the presence of palm oil	Growth in the presence of palm oil and yeast extract
BI1	+	++
BI2	++	++++
BI3	+	++
BI4	+	++
BI 5	++	++++
BI6	+	++
BI7	+	++
BI8	+	++

Bacterial isolates	Growth in the presence of palm oil	Growth in the presence of palm oil and yeast extract
BI9	++	++++
BI10	++	++++
BI11	+	++
BI12	+	++
BI13	++	++++
BI14	+	++
BI15	+	++
BI16	+	++
BI17	+	++
BI18	+	++
BI19	+	++
BI20	+	++
BI21	+	++
BI22	++	++++
BI23	+	++
BI24	+	++
BI25	+	++
BI26	+	++
BI27	+	++
BI28	+	++
BI29	++	++++
BI30	+	++
BI31	++	++++

+ = growth; ++ = growth with high load; ++++ = growth with higher load.

3.5.2. Biodegradability Test of Solid Sludge from Palm Oil Extraction

The growth kinetics of the isolates in the presence of solid sludge from palm oil extraction as the sole source of carbon and energy was measured after 7 days (Figure 4). Through the results obtained, we see that the bacterial strains studied show 5 growth phases. An acceleration phase between D0 and D1, a slowdown phase between D1 and D3, an exponential growth phase between D3 and D5, a decline phase between D5 and D6, then a second acceleration phase between D6 and D7. The growth curves in Figure 4 show that the growth speed of the isolates and consequently that of the degradation of palm oil extraction sludge is very significant during the first 5 days. This growth is continuous during the first 5 days of incubation with a maximum number of microorganisms on the 5th day of incubation in all isolates. The results of optical density (OD) show that all bacterial isolates tested have the ability to use palm oil sludge as the sole source of carbon and energy, but to different degrees. Bacterial isolates BI5, BI31 and BI29 exhibit the highest growth rates, respectively. Bacterial isolate BI9 presents the lowest growth rate. We also note that most of the strains show good growth over the entire duration of the incubation and this growth appears stationary. Nevertheless, we observed a drop in growth between day 5 and day 6, then a recovery between day 6 and day 7 in all isolates.

The results of the evolution of the biomass of the cell suspension of each isolate inoculated in the liquid MSM medium supplemented with 4% stock solution of palm oil extraction sludge after 5 days of incubation are presented in the Figure 5 below. Analysis of variance of the growth rate of the different isolates showed a significant difference ($p = 0.013$). These results showed a variation in the biomass of the isolates between 16 and 187.2%. Bacterial isolates BI2, BI5, BI31 and BI10 showed the highest growth rates. Bacterial isolates BI29 and BI22 showed the lowest growth rates. 75% of isolates had a growth rate greater than 50%.

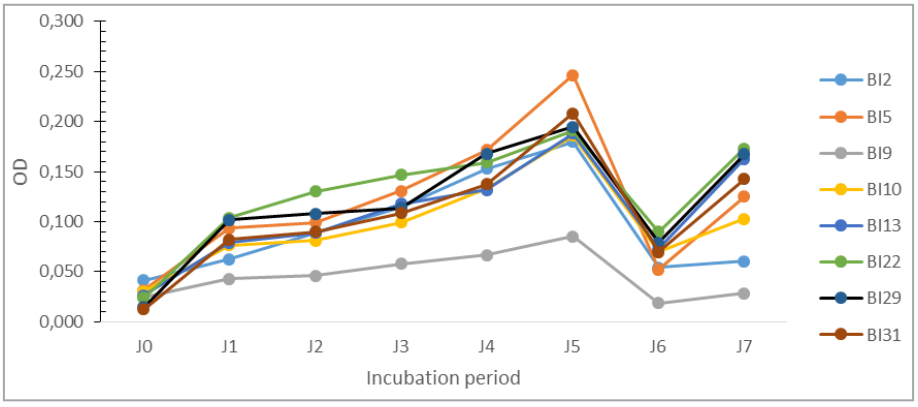

Figure 4. *Growth kinetics of the different strains in the presence of solid sludge from palm oil extraction as the sole source of carbon and energy.*

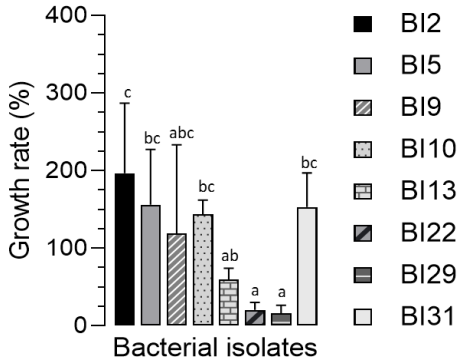

Figure 5. *Growth rate of the 8 bacterial strains after 5 days of incubation in the MSM medium supplemented with 4% stock solution of palm oil extraction sludge.*

Means with the same letters are not significantly different at $P < 0.05$ according to the Duncan test.

4. Discussion

4.1. Evaluation of Hydrogen Potential

The sludge sample studied for microbiological analysis has a slightly alkaline pH of 7.3 after pH meter stability which lasted nearly 30 minutes. This pH is between 6.5 and 8, the pH interval where biodegradation takes place [10]. This pH, close to neutrality, would promote the growth of bacteria and therefore allow the biodegradation of fatty acids to take place efficiently. These results are similar to those obtained by Goma-Tchimbakala *et al.* (2020) who found PH values of 7.02 and 7.32 in two soils polluted by used motor oil [10].

4.2. Isolation, Purification and Storage of Bacterial Isolates

The results of the enumeration on nutrient agar obtained for the different dilutions after 48 hours of incubation showed that the sample of palm oil extraction sludge stored at the SOCAPALM landfill contains a fairly significant bacterial load with a value around 12.6.109 CFU/g of sludge. This high bacterial load is believed to be due to the adaptation of bacteria to the environmental conditions of the environment and the use of palm oil sludge as a source of carbon and energy. It has been proven that the development of microorganisms depends on the characteristics of the environment and the nature of the waste found there [20]. In addition, the adaptation of microorganisms is the consequence of three interdependent mechanisms, namely, the induction and/or depression of specific enzymes, genetic mutations and selective enrichment [21]. Similar results were found by Selmoun *et al.* (2016) from oil drilling waste sloughs, and by Benyahia and Ayadi (2012) from a site polluted by oil [15, 22]. Also, Lebonguy (2019) counted $1.53.10^7$ CFU/g of soil in the polluted soil of a fuel distribution station in Pointe-Noire [23].

Based on all the cultural characteristics, 31 bacteria were isolated and purified from the sample of solid sludge from palm oil extraction. These results show a high culturable bacterial diversity in this waste. This high diversity could be explained by the easier adaptation of bacteria to the carbon source contained in this waste (fatty acids), which are less recalcitrant than other pollutants such as polycyclic aromatic hydrocarbons. These results are in agreement with those obtained by Djien *et al.* (2021) in soil polluted by liquid effluents from an artisanal palm oil extraction factory [24]. Furthermore, other authors such as Ndzobo *et al.* (2022) after the similar study carried out on the isolation, screening and biochemical identification of bacteria with purifying potential in soil polluted by effluents from a paint manufacturing industry, isolated and purified 35 bacterial isolates [18].

4.3. Cultural and Cellular Characterization of Bacterial Isolates

All the macroscopic and microscopic characteristics stud-

ied on the 31 isolates confirm the significant bacterial diversity present in the sludge sample, already shown by the isolation and purification of the colonies. These cultural and cellular characteristics of the isolates allow us to have a first idea of the type of bacteria. All 31 bacterial isolates are bacilli, including 12 Gram+ bacilli and 19 Gram- bacilli. According to the literature, bacteria from the bacilli group are the most involved in the biodegradation of pollutants. These results corroborate those of Goma-Tchimbakala *et al.* (2020) who found that the bacteria isolated from soil polluted by polycyclic aromatic hydrocarbons all belonged to the *Bacillus* genus [10]. Also Xingjian *et al.* (2018) found that among bacteria isolated from oil-polluted soil in China, most were bacilli [25].

The hierarchical classification of isolates obtained on the basis of cultural characteristics made it possible to distinguish two large groups of bacteria. Group 1 consists of 19 bacterial isolates which are grouped into three subgroups. Group 2 consists of 12 bacterial isolates which are also grouped into three subgroups. The grouping of the isolates into several groups further shows the existence of the high diversity within the isolates in the sample. Similar results were found by Benadjila (2017) who obtained a significant diversity of bacteria in soil polluted by hydrocarbons [26].

4.4. Biochemical Characterization of Bacterial Isolates

4.4.1. Characterization of Gram+ Bacteria

All the cultural, cellular and biochemical characteristics led to the identification of bacterial isolates BI1, BI2, BI3, BI4, BI8, BI14, BI15, BI16, BI18, BI19, BI21 and BI26 as belonging to the genus *Bacillus sp*. This result could be justified by the fact that bacteria of the genus *Bacillus sp*. are a group of bacteria very involved in the natural biodegradation of pollutants. These results are in agreement with those of Benyahia and Ayadi (2012) who found during their work on the characterization of the microbial flora of waste sludge from RTC-SONATRACH oil tanks that *Bacillus sp*. represented 30% of all microorganisms isolated [22]. These results also corroborate those obtained by Djien *et al.* (2021) who found, during their studies on the isolation of bacteria with purifying potential and application to the treatment of effluents from an artisanal palm oil mill in the coastal region of Cameroon, bacteria of the genus *Bacillus sp* [24].

4.4.2. Characterization of Gram- Bacteria

The results of the cultural, cellular and biochemical characteristics led to the identification of bacterial isolates BI6, BI7, BI9, BI12, BI20, BI22, BI27, BI28, BI29 and BI30 as belonging to the genus *Pseudomonas sp*. However, these characters led to the identification of bacterial isolates BI5, BI10, BI11, BI13, BI23, BI24, BI25 and BI31 as belonging to the species *Proteus mirabilis* and isolate BI17 as belonging to the species *Klebsiella pneumoniae*. The presence of these species in polluted environments is due to their ability to tolerate and use pollutants as a source of carbon and energy [27]. Most of these strains have already been identified in environments polluted by hydrocarbons [10, 27-29]. Liang *et al.* (2014) also identified a strain of *Pseudomonas sp*. JP1 of polluted sediments from Shantou port in China [27].

4.5. Biodegradability Test of Palm Oil and Extraction Sludge Using Bacterial Isolates

4.5.1. Palm Oil Biodegradability Test

The results of the palm oil biodegradation test show that all 31 bacterial isolates tested grew on the M2 medium supplemented with 2% palm oil and devoid of yeast extract after 48 hours with a difference in the load. Unlike, in boxes containing M2 medium with 2% palm oil and 1g/L of yeast extract, growth is obtained after 24 hours with a heavier load. These observations show that all bacteria isolated from palm oil extraction sludge can use the palm oil contained in this sludge as a sole source of carbon and energy and therefore can degrade this pollutant. The presence of a second carbon source leads to more rapid growth of these bacteria which can effectively degrade this waste. Similar results were found by Selmoun *et al.* (2016) during their work on the contribution to the study of the biodegradation of hydrocarbons in drilling muds by bacteria producing biosurfactants [15]. Benyahia and Ayadi (2012) found during their work on the characterization of the microbial flora of waste sludge from RTC-SONATRACH oil tanks that half of the strains isolated had good growth on all the hydrocarbons tested as carbon substrate [22].

4.5.2. Biodegradability Test of Solid Sludge from Palm Oil Extraction

The growth kinetics measured after 7 days for the 8 isolates in the presence of solid sludge from palm oil extraction as the sole source of carbon and energy showed 5 growth phases. An acceleration phase between day 0 and day 1 during which there is an acceleration in the rate of bacterial division. A slowdown phase between D1 and D3 during which there is a slowdown in the rate of bacterial division. A phase of exponential growth between D3 and D5 during which the bacteria are in their optimum physiological and metabolic state with maximum division. A phase of decline between D5 and D6 where growing conditions become unfavorable. Then a second acceleration phase between D6 and D7. The absence of the latency phase shows that the latency time was very short. These results demonstrate a more or less rapid adaptation of the isolates to the carbon source used and the rapid establishment of the enzymatic machinery necessary for the biodegradation of this waste. The resumption of the acceleration of growth between day 6 and day 7 would be due to the fact that certain bacteria have adapted to consume

the waste generated or their dead congeners. The growth curves in Figure 4 show that the speed of growth and therefore that of the degradation of palm oil extraction sludge is very important during the first 5 days. This growth is continuous during the first 5 days of incubation with a maximum number of microorganisms on the 5th day of incubation in all isolates. The optical density results show that all bacterial isolates tested have the ability to use palm oil sludge as the sole source of carbon and energy, but to different degrees. These results could be justified by the fact that the initial attack on the carbon source differs from one isolate to another and, moreover, by the individual growth characteristics of each isolate [30]. Bacterial isolates BI5, BI31 and BI29 exhibit the highest growth rates, respectively. Isolate BI9 presents the lowest growth rate. We also note that most of the isolates show good growth over the entire duration of the incubation, their growth appears stationary. However, we observed a drop in growth between day 5 and day 6, then a recovery between day 6 and day 7 in all isolates. These results corroborate those obtained by Guermouche (2014) who, during their work on the molecular characterization of bacteria involved in the biodegradation of hydrocarbons, found that certain bacterial strains had more capacity to degrade oil compared to others [30].

The results of the evolution of the biomass of the cell suspension of each isolate inoculated in the liquid MSM medium supplemented with 4% stock solution of palm oil extraction sludge after 7 days of incubation showed a variation of the biomass of the isolates between 16 and 187.2%. Bacterial isolates BI2, BI5, BI31 and BI10 showed the highest growth rates and isolates BI29 and BI22 the lowest growth rates. Seventy-five percent of the isolates had a growth rate greater than 50%. These results corroborate those of Ndzobo *et al.* (2022) who found during their studies on the isolation, screening and biochemical identification of bacteria with purifying potential in a soil polluted by the effluents of a paint manufacturing industry in Cameroon that the isolates IS20, IS59, IS19; isolates IS25, IS35, IS45, IS37, IS55 and isolates IS06, IS08, IS41, IS43, IS15, IS30, IS49 and IS10 had the growth rates of 37%, 50% and 30%, respectively [18].

5. Conclusion

This work aimed to isolate and characterize indigenous bacteria with biodegradation capacities in sludge from palm oil extraction. It appears that 31 bacteria were isolated and purified from the sludge sample, including 12 *Bacillus sp*, 10 *Pseudomonas sp*, 8 *Proteus mirabilis* and 1 *Klebsiella pneumoniae*. All 31 bacterial isolates tested for their ability to use palm oil as the sole carbon and energy source grew in the culture medium in the presence of palm oil as the sole carbon and energy source. All the top 8 isolates selected from the palm oil biodegradability test and tested for their ability to degrade solid sludge from palm oil extraction showed very rapid adaptation to the source of carbon, a very high speed and growth rate. Of all these 8 bacterial isolates, bacterial isolates BI2, BI5, BI31, BI10 and BI9 showed the highest growth rates. These isolates could be used to constitute consortia of microorganisms that can be used in the treatment of waste generated by palm oil extraction.

Abbreviations

ADH: Arginine Dihydrolase
AMY: Amygdalin
ARA: Arabinose
BAG: Sucrose
BI: Bacterial Isolate
CIT: Citrate
GEL: Gelatin
GLU: Glucose
H_2S: Hydrogen Sulfide
IND: Indole
INO: Inositol
LDC: Lysine Décarboxylase
MAN: Mannose
MEL: Melibiosis
OD: Optical Density
ODC: Ornithine Décarboxylase
ONPG: O-nitrophényl- D-galactopyranoside
OX: Oxydase
RHA: Rhamnose
SOR: Sorbitol
TDA: Tryptophan Deaminase
URE: Urea
VP: Voges-Proskauer

Acknowledgments

The authors thank the Laboratory of the Department of Plant Biology of the Faculty of Science of the University of Douala for some analyzes that have been done.

Author Contributions

Tassong Saah Denis: Data curation, Software, Funding acquisition, Investigation, Methodology, Writing – original draft

Simo Claude: Conceptualization, Software, Formal Analysis, Validation, Visualization, Writing – original draft, Project administration, Writing – review & editing

Taffouo Vctor Désiré: Supervision, Validation

Conflicts of Interest

The authors declare no conflicts of interest.

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