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Isolation, Screening and Biochemical Identification of Bacteria with Purifying Potential in the Effluents of a Paint Manufacturing Industry

Emmanuel Joël Ndzobo Ndzana[1, *], Marie Frederic Tavea[1], Josiane Bella[1], Romeo Tagnikeu Fobasso[1], Wilfred Atangana[1], Joel Styve Tcheugoue[1], Angelin Yanick Kuissie[1], Felecite Njen[1, 3], Regis Tanga Tanga[1], Youagang Gougueu Harris Stephane[4], Emmanuel Mpondo Mpondo[2]

[1]Biochemistry Laboratory, Faculty of Science, University of Douala, Douala, Cameroon

[2]Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Douala, Cameroon

[3]Institute of Agricultural Research for Development (IRAD), Yaounde, Cameroon

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

Email address:

ejndzobondzan@gmail.com (E. J. N. Ndzana)

[*]Corresponding author

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Abstract: The technology based on the use of micro-organisms, in this case bacteria isolated from sites contaminated by industries, represents an alternative option for the treatment of wastewater before discharge into the environment. To this end, we carried out research in the city of Douala on the isolation, selection and identification of indigenous bacteria, potential for the bio-purification of effluents from a paint manufacturing industry located in the Logbaba industrial zone (Douala Cameroon). The bacteria were isolated from soils contaminated by effluents from the said industry. A programme of three selection tests in mineral saline liquid medium was implemented for the choice of isolates. The selection criterion was based on the growth of the isolates as assessed by OD measurement with a spectrophotometer (BIOBASE) at 600 nm. At the end of the selection process, 05 isolates (IS10, IS20, IS25, IS35 and IS41) were considered to be the best potential candidates for effluent bio-purification. On the basis of a pre-test identification completed by the API20E system, they were identified as *Stenotrophomonas maltophilia* (IS41); *Pseudomonas luteola* (IS20 and IS35); *Pseudomonas fluorescens/putida* (IS25) and *Yersinia pseudotuberculosis* (IS10) Exploiting the bio-purification capacities of these bacteria in the context of biological treatment of these polluting aqueous discharges could contribute to the preservation of the environment and human health. In the course of our further work, the bio-purification potential of these isolates will be evaluated.

Keywords: Isolation, Isolate, Identification, Effluent, Scrubber, Paint

1. Introduction

Industrial effluents are a major source of direct and often continuous input of pollutants into aquatic environments, with long-term implications for ecosystem functioning [1]. Environmental degradation due to the discharge of these effluents is a real problem in several countries. This situation is even more serious in developing countries where these waters are poorly treated or not treated before discharge [2]. In Cameroon, the paint production industry is a major water consumer and owner of huge volumes of wastewater. It generates wastewater from equipment washing operations, accidental spills and batch spills that do not meet specifications. These effluents often contain precursor paint

components at appreciable carbon concentrations [3]. Many of the chemicals used in the production of paints are responsible for high concentrations of organic and inorganic toxic pollutants [4], colored materials and hazardous pollutants such as heavy metals [5]. Toluene and xylene are organic pollutants whose presence in these effluents is often mentioned in the literature. On the other hand, the release of these effluents into the environment without prior treatment could cause health problems, ecological imbalance and bioaccumulation in aquatic organisms. Thus, in view of the pernicious effect of these effluents on the environment, remediation strategies are envisaged to prevent the contamination of soil and surface water. Various physico-chemical processes such as ultra violet oxidation, fixation and flocculation are practiced but present several drawbacks including cost, complexity and in many cases, do not completely destroy the contaminating compounds [6]. For these reasons, the bio-remediation approach, which exploits the natural capacities of microorganisms to degrade organic pollutants, seems to be an environmentally and socially acceptable alternative. It is therefore important to explore the possibility of isolating strains of bacteria able of degrade these effluents. However, in soils contaminated by these effluents, there are indigenous, heterogeneous bacteria whose metabolic capacities can be exploited in the treatment of these effluents. Hence the present study, which aims to isolate, select and identify bacterial isolates in soils contaminated by effluents from a paint manufacturing industry. The isolation of these strains could contribute to fix the problem of environmental pollution through a bio-treatment application of these effluents.

2. Materials and Methods

2.1. Sampling

2.1.1. Soil Sampling

Soil collection was carried out outside a paint production factory that has been established for more than three decades in the Logbaba industrial zone (Lat: 04°02'06,6"N; Long: 009°44' 48,3"E) of the third district of Douala. Soils were collected at 5 points at a depth of about 10 cm [7, 8] after removal of the surface layer in places visibly stained by industrial discharges. Soils collected with a hand trowel were immediately put into small sterile plastic bags and transported to the laboratory. Once in the laboratory, a composite sample was made in a sterile glass bottle and stored at 4°C [9] for further work.

2.1.2. Sampling of Effluents

The effluent was collected by trapping it in a sterile 15L bucket at the outlet of a drainpipe and then transferring it to a rinsed 1L polystyrene bottle. Once in the laboratory, the effluent was treated with sulphuric acid and stored at 8°C.

2.2. Isolation, Purification and Macroscopic Screening

2.2.1. Plating

Bacteria were isolated by the serial dilution method and inoculated into an effluent-contaminated composite soil sample suspension. To prepare the soil suspension, 10 g of composite soil sample was dissolved in 90 ml of sterile distilled water contained in a 250 mL Erlenmeyer flask [10]. This was incubated for 48 hours [7] under stirring (200tr/min) at a temperature of 30°C. 1 ml of the incubated soil suspension was taken with a micropipette and diluted in a tube containing 9ml of previously sterilized distilled water. Then, a series of dilutions was performed from the first dilution 10^{-1} to 10^{-8}. 100µl of the dilutions 10^{-3}; 10^{-5} and 10^{-7} were each seeded by spreading on nutrient agar [11, 12] and then incubated at 37°C for 24 hours.

2.2.2. Isolation, Purification and Macroscopic Screening

Colonies of well-differentiated bacteria were coded and subjected to successive replicates on nutrient agar and re-incubated at 37°C for 24 hours [13]. Each series of replicates was preceded by macroscopic screening based on cultural characters (colony appearance; color and metabolite production etc) to eliminate cases of redundant isolates. The selected pure isolates were kept on slant agar at 4°C [2] for further work.

2.3. Selection Tests for Potential Bio-purifying Isolates

Selection tests were carried out in liquid MSM supplemented iteratively by combining various protocols [14, 9].

2.3.1. Selection Test in Liquid Mineral Salt Medium (MSM) Supplemented with a Paint Suspension

Three test sequences were carried out under similar conditions to verify the ability of the 35 purified isolates to use the organic compounds in the paint as their sole source of carbon and energy.

Two paints used as a constituent of the stock paint solution are produced by the same industry and marketed in Cameroon. 50g of matte acrylic paint and 10g of glycerophthalic-based oil paint (Email LUCIDO) were mixed and homogenized and left to stand in the dark for a week. 10g of this mixture was dissolved in 1L of distilled water to form the stock solution.

Three pre-culture sequences were performed according to the protocol of Temesgen et al. [15] whose nutrient broth composition in grams per liter (g/l) was: glucose: 3; NH_4Cl: 3.2; KH_2PO_4:1; K_2HPO_4:10; $MgSO_4 \cdot 7H_2O$: 0.1; NaCl: 5 and peptone: 5. Each volume of broth was distributed at a rate of 50 mL in 100 mL glass vials. All flasks were autoclaved at 121°C for 15 minutes [2]. Each pure 24-hour isolate collected on nutrient agar with a sterile loop was inoculated into a vial. The set of flasks was incubated in a shaker at 32°C for 48 hours.

The liquid MSM used had a salt composition similar to that of the pre-culture broth but free of glucose and peptone. Three volumes of 4% (v/v) supplemented liquid MSM, 506mL, 644mL and 598mL corresponding to a test series, were prepared by adding adequate masses of salts in a 1L Erlenmeyer flask. The pH of each MSM solution was

adjusted to 7.0 ±2 by adding 1M NaOH or 1M HCl [2] and the solutions homogenized by manual shaking for two minutes. 45ml of each supplemented MSM was dispensed in duplicate into 100ml glass vials and the whole sterilized by autoclaving at 121°C for 15 minutes [14].

For each test series, pre-cultures of the isolates were centrifuged at 4000rpm for 10min [9], the resulting pellets were washed twice with 0.9% NaCl solution and centrifuged again at 4000rpm for 10min. The pellets recovered after centrifugation were each suspended in 20 mL of sterile 0.9% NaCl solution and calibrated to OD: (0.8±0.03); (0.5±0.02) and (0.4±0.01) in the spectrophotometer at 600nm [22]. In each test series, 5 mL of calibrated cell suspension from each isolate was inoculated in duplicate into 100mL vials containing 50 mL of supplemented MSM. A sterile supplemented MSM vial not inoculated with bacterial suspension into which 5mL of 0.9% NaCl was introduced served as a control. OD measurements of the test vials against the control vial before and after incubation for 7 days in a shaker at 30°C [14, 18] were conducted. The OD measurements were used to determine the growth rate of each isolate according to the formula:

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

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

Selected isolates were streaked again on nutrient agar.

2.3.2. Selection Tests for Isolates in MSM Supplemented with Effluent

Two independent growth tests were conducted using effluent as the sole carbon and energy source for the isolates. The experimental effluent used was a mixture of four effluent collections at different days. The first test, carried out in the presence of the experimental effluent diluted to 10%(v/v), was designed to confirm the ability of the isolates to consume the effluent molecules as a source of carbon and energy. However, the second test aimed to select from isolates selected in the first test in the diluted experimental effluent those that could tolerate the effluent highly loaded with organic pollutants. A similar protocol was applied to both tests. In the first test, 150mL of raw experimental effluent filtered on cotton contained in a coffee filter paper was added to 1350 mL of previously prepared distilled water-based liquid MSM. 80mL of the resulting solution was dispensed into 100 mL bottles and the whole set was autoclaved at 121° for 15 minutes. Cell suspensions of the 16 selected isolates in the presence of paint solution were prepared from the 48 hours old isolates collected on nutrient agar. The suspensions were calibrated in a spectrophotometer at 600nm at four OD values, (0.3±0.03) for IS20; IS19; IS26; IS08; IS37; (0.1±0.012) for IS10; IS35; IS41; IS59; IS15 and IS25; (0.4±0.01) for IS55; IS45 and IS49 and (0.5±0.03) for IS06; IS43 and IS30. Subsequently, 8mL of each calibrated cell suspension was inoculated into 80mL of sterile liquid MSM supplemented with the diluted experimental effluent. In parallel, a control flask was made. Prior to incubation in a shaker at 32°C, a double UV-visible spectrophotometer absorbance reading [19] of the test vials against the control was taken. Growth monitoring by OD measurement at regular 24-hour intervals for six days was performed. At the end of this test, 8 isolates were retained and in the second test selected from the raw test effluent. For the second test, suitable masses of salts were added to 800mL of filtered raw experimental effluent contained in a 1L Erlenmeyer flask and then dispensed at a rate of 40mL into 100mL glass vials. All flasks were autoclaved. 4mL of cell suspension of isolates, IS20; IS10; IS35; IS41; IS25: IS45 IS49 and IS06 calibrated to OD (0.8 ± 0.02) were inoculated in duplicate into sterile 100mL vials and their OD measured against the non-inoculated control vial before incubation at 32°C in a shaker under static conditions. Regular monitoring of isolate growth by OD measurement was performed every 32 hours.

2.4. Identification of Five Selected Bacterial Isolates

The 5 isolates were pre-identified and complemented with API20E biochemical tests. The pre-identification allowed partial definition of morphological (appearance, shape and outline) and biochemical characters such as catalase reaction [20], oxidase reaction and Gram staining. Interpretation of the API20E biochemical tests generated numerical codes that allowed the identification of isolates through the API Web.

2.5. Statistical Analysis

ODs obtained from growth monitoring during the different selection tests were recorded in Microsoft Excel table and analyzed using SPSS16.0. Histograms were plotted by GraphPad Prism 5.0 and Excel. Comparison of means was performed using Duncan test and ANOVA at 5% of average probability.

3. Results

3.1. Isolation, Purification and Macroscopic Screening of Isolates

Sixty bacterial isolates were isolated from the contaminated soil samples and subjected to purification and macroscopic screening on nutrient agar. At the end of this process, 35 pure isolates were selected for screening. These were: IS39, IS05, IS46, IS06, IS49, IS30, IS08, IS43, IS27, IS15, IS10, IS41, IS37, IS47, IS35, IS25, IS17, IS40, IS44, IS45, IS55, IS31, IS04, IS13, IS24, IS18, IS11, IS07, IS02, IS14, IS16, IS19, IS26, IS59 and IS20.

3.2. Selection Tests for Isolates with the Best Bio-purification Potential

3.2.1. Selection Test of Isolates in Liquid MSM Supplemented with a Paint Suspension

The results of the evolution of the biomass of the cell suspensions inoculated in the MSM supplemented with paint solution after incubation for 5 days are presented below.

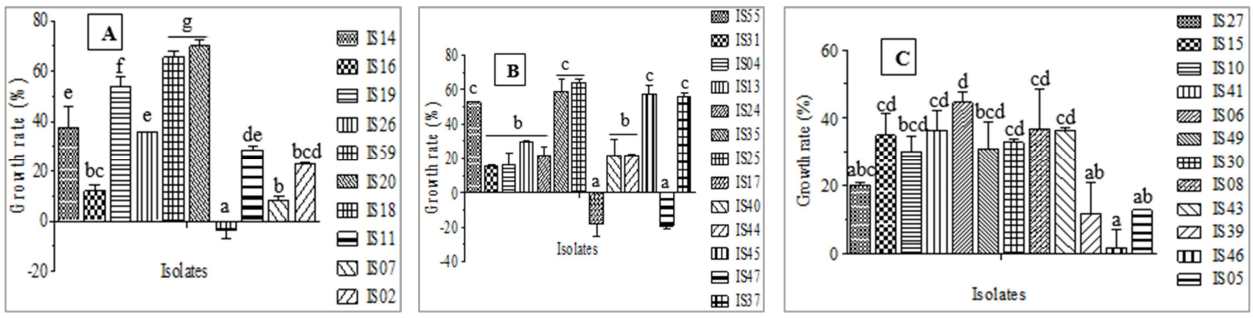

Figure 1. *Growth rates of 35 bacterial isolates after 5 days incubation in liquid MSM supplemented with paint solution (10g/L). A: serial 1; B: serial 2; C: serial 3. Means with the same letters are not significantly different by the Duncan test at 5% of average probability.*

The overall analysis of these results showed a biomass variation of the isolates ranging from -18 to 65%. Three isolates, IS26 (Series I); IS17 and IS47 (Series II) showed a negative biomass variation. 16 isolates including 3 isolates from series I (IS20; IS59 and IS19); 5 from series II (IS25; IS35; IS45; IS37 and IS55) and 8 isolates from series III (IS06; IS08; IS41; IS43; IS15; IS30; IS49 and IS10) with biomass levels above 37%; 50% and 30% respectively were selected for further testing.

3.2.2. Selection Test of Isolates in the Presence of Effluent

Two tests were carried out in the presence of a composite effluent. The first test was carried out in liquid mineral medium supplemented with 10% (V/V) effluent to confirm the growth of the 16 isolates from the first selection test in the presence of the paint suspension. The results of the daily growth monitoring of the isolates for six days are shown in Figure 2 below:

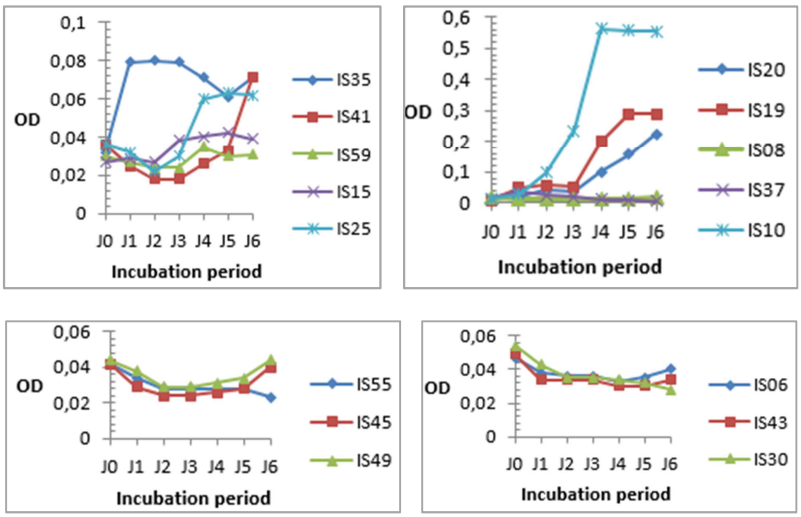

Figure 2. *Growth rates of 16 bacterial isolates subjected to the liquid MSM selection test supplemented with 10% (v/v) raw effluent.*

Analysis of the growth profiles of the isolates showed a range of growth amplitudes with various lag times of 0 and 6 days. Isolates calibrated at OD (0.4±0.01) and (0.5±0.036) showed a lag time of approximately five days (IS55 and IS45) and (IS06 and IS43). For isolates calibrated at OD (0.1±0.012) this time could go beyond 6 days for IS37 and IS08. 8 isolates were selected according to the degree of biomass on day 6 and streaked on nutrient agar for further work on nutrient agar. These were the isolates, IS25; IS35 and IS4 (OD≥0.06) from group A; IS10 and IS20 (OD≥0.28) from group B; IS45 and IS49 (OD≥0.04) from group C and IS06 (OD≥0.04) from group D. The second selection test of the 8 isolates in the mineral composite raw effluent was among to assess the ability of the isolates to tolerate the imposed experimental conditions. The results of the monitoring of the growth of the isolates measured at regular intervals of 32 hours during a period of 12 days of incubation are shown in the graph below:

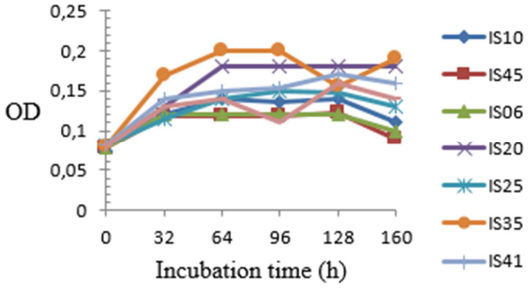

Figure 3. *Tolerance test of 08 bacterial isolates in experimental raw effluent.*

By examining the growth profiles, it appears that all 08 isolates develop tolerance to the composite raw effluent with varying growth capacities. Strong growth was expressed by

isolate IS35 with a tolerance time of 96 hours. To complete the selection process, isolates with a tolerance time greater than or equal to 96 hours with an OD at the entrance to the stationary phase greater than or equal to 0.14 were selected. Thus, 5 isolates, IS10; IS20; IS25; IS35 and IS41 were identified as the best potential candidates for biodegradation of effluents from a paint manufacturing industry.

3.3. Identification of Isolates

The isolates, IS10, IS20, IS25, IS35 and IS41 considered as potential strains for effluent bio-purification were subjected to a preliminary identification of morphological and enzymatic characteristics (oxidase and catalase) and completed by the API20E. The results obtained show that the 5 isolates had rounded, milky white colonies; Gram negative with bacillary morphology and positive to enzymatic tests (oxidase and catalase). Analysis of the numerical profiles generated by API Web software identified the isolates as *Yersinaia pseudotuberculosis* (IS10); *Pseudomonas luteola* (IS35 and IS20); *Pseudomonas fluorescens/putida* (IS25) and *Stenotrophomonas maltophilia*. The identification results are presented in Table 1 and Figure 4 below:

Table 1. *Morphological and enzymatic characters of the 05 selected bacterial isolates.*

Isolates	Macroscopy		Microscopy		Enzymatic tests		API20E	
	Colour	S	G	S	Cat	Ox	Codes	Species
IS10	white	round	-	rod	+	+	1016153	*Y. pseudotuberculosis*
IS20	white	round	-	rod	+	+	3003000	*P. luteola*
IS25	milky white	round	-	rod	+	+	2003000	*P. fluorescens/putida*
IS35	milky white	round	-	rod	+	+	2201021	*P. luteola*
IS41	milky white	round	-	rod	+	+	1002000	*S. maltophilia*

Y: *Yersinaia*; P: *Pseudomonas*; S: *Stenotrophomonas*; Cat: Catalase; Ox: Oxydase; G: Gram staining; S: Shape; +: Positive; -: Negative.

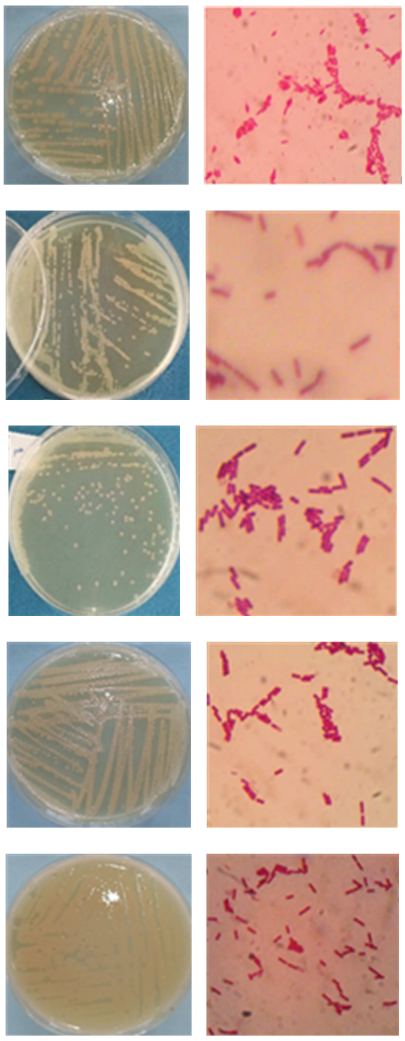

Figure 4. *Isolates IS41, IS35, IS25, IS20 and IS10 (left side: culture on nutrient agar; right side: ngeative Gram staining).*

4. Discussion

4.1. Isolation, Macroscopic Screening and Purification

Microbial diversity is the most extraordinary reservoir of life in the biosphere that we have only recently begun to explore and understand. Over time, microorganisms adapt to various environmental extremes by developing new metabolic pathways [21]. In this work, 60 strains of bacteria were isolated on nutrient agar and 35 were retained after successive subculturing coupled with macroscopic screening for selection tests. The bacteria were isolated from soil samples contaminated by effluents from a paint manufacturing industry that has been established for more than three decades in the BASSA industrial zone (Douala-Cameroon). The bacteria isolated in these contaminated soils would have undergone an adaptation to the polluting molecules of the paint manufacturing industry effluent. It has been proven that the development of microorganisms depends on the characteristics of the environment and the nature of the waste that flows [22]. Also, the phenomenon of adaptation is described as the consequence of three interdependent mechanisms, induction and/or depression of specific enzymes; genetic mutations and selective enrichment [23].

4.2. Selection of Potential Biodegradation Isolates from the Effluent

Various techniques for evaluating the biodegradation activity of an organic pollutant by microorganisms [16, 17], in this case bacteria, are found in the scientific literature. However, many research works are based on the criterion of growth in supplemented liquid MSM to highlight the capacity of bacteria to use an organic pollutant as a source of carbon and energy. The three selection tests in supplemented liquid

MSM helped to highlight the bio-purifying potential of the isolates, but also to select them. In the first selection test, the results of the biomass variation of 35 isolates after incubation for 5 days are shown in figure 1. A holistic analysis of the results showed a biomass variation of -18 to 65%. 3 isolates, IS18; IS17 and IS47 had negative growth rates and the rest of the isolates showed a positive growth rate. The negative growth rates observed would reflect the inability of these three isolates to adapt to the experimental conditions, including the inability to consume the organic molecules supplied by the paint solution. On the other hand, the rest of the isolates that showed a positive growth rate would use the organic molecules of the experimental media as their only source of carbon and energy, which would explain the increase in their biomass after incubation. For further work, sixteen isolates, IS20; IS59 and IS19 with growth rates higher than 37% (serial I); IS25; IS35; IS45; IS37 and IS55 with growth rates higher than 50% (serial II) and IS06; IS08; IS41; IS43; IS15; IS30; IS49 and IS10 with growth rates above 30% (serial III) were selected and streaked on nutrient agar for a second screening in liquid MSM supplemented with 10%(v/v) of the composite effluent. The results of growth monitoring of calibrated cell suspensions of the 16 isolates tested in MSM supplemented with 10%(v/v) of the experimental raw effluent in test vials are presented in Figure 2. Analysis of these results showed growth profiles of isolates with lag times fluctuating from zero to six days. These lag times correspond to the time required for each isolate to adapt to the experimental environment and to synthesize the enzymes required for the degradation of the organic pollutants brought by the effluent. The growth observed would be linked to the use of the organic molecules contained in the effluent as the only source of carbon energy. 8 isolates were selected according to the level of biomass on day 6 for testing in the presence of the experimental raw effluent. These were the isolates, IS25; IS35 and IS4 (OD≥0.06) from group A; IS10 and IS20 (OD≥0.28) from group B; IS45 and IS49 (OD≥0.04) from group C and IS06 (OD≥0.04) from group D. As for the test conducted in the experimental raw effluent, the results of growth monitoring of cell suspensions calibrated at OD (0.6±0.02) and inoculated in duplicate in flasks are shown in Figure 3. Examining the growth profiles, it is apparent that all 08 isolates developed growth in the presence of the experimental raw effluent with varying growth capacities. Isolate IS35 expressed strong growth with a survival time of 96 hours. Conversely, Isolates IS06 and IS41 showed poor growth with survival times of 128 hours. To complete the selection process, isolates with a tolerance time greater than or equal to 96 hours with an OD value at the entrance to the stationary phase greater than or equal to 0.14 were selected. Thus, 5 isolates, IS10; IS20; IS25; IS35 and IS41 were judged to have the ability to tolerate the highly loaded effluent and were considered to be the best potential candidates for biodegradation of effluents from a paint manufacturing industry.

4.3. Identification of the Selected Isolates

The identification results presented in Table 1 and Figure 4 reveal that all 5 isolates had rounded and milky white colonies. Light microscopic observation after Gram staining showed that the isolates were Gram negative bacilli and positive for enzyme tests (oxidase and catalase). The oxidase positive isolates produce cytochrome c oxidase, an enzyme in the electron transport chain of bacteria and this suggests that they are aerobic [24]. The API20E system through numerical profile analysis by API Web software identified: IS10 (1016153) as *Yersinaia pseudotuberculosis*; IS35 (2201021) and IS20 (3003000) as *Pseudomonas luteola*; IS25 (2003000) *Pseudomonas fluorescens/putida* and IS41 (1002000) as *Stenotrophomonas maltophilia*. These isolates belong to the phylum Proteobacteria of the class Gammaproteobacteria [25]. The API20E system is one of the most commonly used bacterial identification techniques by many researchers. It has been used by, Ida *et al*. [26] to identify *Pseudomonas luteola*; Liu M *et al*. [10] in the identification of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens/putida*, *Pseudomonas luteola* and *Stenotrophomonas maltophilia*. However, API 20E has limitations due to subjective interpretation of biochemical reactions.

5. Conclusion

The study aimed at isolating, selecting and identifying bacterial isolates with better biodegradation potential from paint industry effluents. For this purpose, bacterial strains were isolated from samples of soil contaminated with effluents from a paint industry by plating and then purified on nutrient agar. The pure isolates were subjected to three independent selection tests based on growth in supplemented liquid minimal saline medium, a paint suspension, a 10%(V/V) diluted experimental raw effluent and the experimental raw effluent. At the end of the tests, 5 isolates, IS10; IS20; IS25; IS35 and IS41 were selected and identified according to cultural, morphological, enzymatic (catalase and oxidase) and biochemical characters by API20E. These isolates were identified by API20E as, *Y. pseudotuberculosis* (IS10); *P. luteola* (IS20 and IS35); *Pseudomonas fluorescens/putida* (IS25) and *S. maltophilia* (IS41). The biodegradative potential of paint industry effluents by these identified species will be assessed in further work.

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