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Chromosomal and plasmid mediated degradation of crude oil by *Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria* isolated from the soil

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In this work, the degradative abilities of some bacteria isolated from the soil were studied. Five distinct isolates were subjected to substrate susceptibility test to determine their potential degradative abilities in the presence of different hydrocarbons. The best three crude oil degraders were selected and their identities were further established using API technique. Furthermore, the extent of each degradative ability of the three bacteria was determined using gas chromatography (GC) technique to analyze the residual crude oil after 21 days. The pH and total viable counts (TVC) were also monitored every three days for the period of the study. The three isolates were later subjected to plasmid extraction and the cured isolates were again exposed to crude oil. The initial bacteria isolated were; *Pseudomonas cepacia* (B1), *Micrococcus luetus* (B3) *Bacillus coagulans* (B4), *Citrobacter koseri* (B2) and *Serratia ficaria* (B5). *C. koseri, S. ficaria* and *B. coagulans* had strong growth in crude oil. *B. coagulans* was able to degrade 95% of the crude oil; *S. ficaria* degraded 92% of the crude oil, and *C. koseri* degraded 91% of the crude oil using it as the sole carbon source. The pH was relatively the same throughout the period and the TVC that were initially low for the three bacteria, later increased. Degradative abilities of *B. coagulans* and *C. koseri* were chromosomal mediated while *S. ficaria* was plasmid mediated. The biological/genetic basis of their degradative activities can be used improve their degradative abilities.

Key words: Chromosomal, crude oil, degradation, plasmid, soil.

INTRODUCTION

Plasmid or chromosomal mediated degradation can be useful and effective during the remediation of crude oil contaminated sites. This process offers the advantage of low operating cost and safety among others. These advantages in bioremediation have served as motivation for developing different bioremediation process and also optimizing the existing processes to restore and minimize harm caused by crude oil pollution. Many bacterial strains

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have genetic determinants of resistance/degradative abilities to pollutants. These determinants are often found on plasmids, chromosomes and transposons. These degradative abilities occur in high frequencies with much greater quantitative prominence after pollution (Shukla et al., 2006). There was proposition that the development of resistant/degradative population in a polluted site can lead to gene transfer (vertical or horizontal gene transfer), transposons transfer, plasmids transfer and possible spontaneous mutants due to the presence of pollutants (Kulkarni and Kaliwal, 2015). A plasmid is a DNA molecule that is separate and can replicate independently of the chromosomal DNA. Plasmids usually occur naturally in bacteria, but are sometimes found in eukaryotic organisms (the 2-micrometre-ring in *Saccharomyces cerevisiae*) (Alberts, 2002). Plasmid size varies from 1 to over 1,000 kilobase pairs. The number of identical plasmids within a single cell can range anywhere from one to even thousands under some circumstances (Alcamo, 2000). Plasmids are not usually required by their host cell for its survival. Instead, they carry genes that confer a selective/specific advantage on their host, such as resistance to heavy metals, toxic substances, antibiotic or resistance to naturally made antibiotics carried by other organisms (Anna et al., 2008; Abdel-lateif, 2017). Alternatively, they may produce antibiotics (toxins) that help the host to compete for food or space. Plasmids are sub-grouped into five main types based on phenotypes: Fertility-F-plasmids, virulence plasmids, resistance-(R) plasmids, col-plasmids and degradative plasmid. Degradative plasmid enables the digestion of unusual substances, such as hydrocarbons, pesticides, plastic and other xenobiotics (Kulkarni and Kaliwal, 2015). The degradative ability of a bacteria which could be chromosomal or plasmid mediated can be improved by conducive environment, long time exposure to materials been degraded and producing mutant species or engineered species. Identification of these degradative dependencies (plasmid/chromosome) can be of great help in genetic engineering techniques of bacteria strains with the ability to degrade pollutants. In bioremediation/biodegradation obtaining optimal bio-degradative conditions and best strain selection are of major concern which has to be overcome to have a significant degradative positive result (Thompson et al., 2005; Liu et al., 2016). This research studied the degradative ability of three bacteria isolated from the soil and the biological dependency of their degradative abilities.

MATERIALS AND METHODS

Sources of soil and hydrocarbon used

Soil sample was collected from the Botanical Garden, University of Lagos, Lagos State, Nigeria. The diesel, kerosene, and engine oil used were bought from Total filling station in Ketu, Lagos (6°35`N 3°45`E) and the crude oil (bony light) used was gotten from Shell flow station near Portharcourt (4°49`N 7°2`E).

Determination of soil physiochemical parameters

The pH, moisture content, total phosphate, sodium, potassium, magnesium, calcium and nitrogen were determined using methods described by AOAC (2012).

Conveyer identification of the bacteria isolates

Bacteria isolates were prepared on agar plates and the characteristics of the colonies of the pure cultures were observed and recorded. Subsequently, biochemical tests were carried out on the bacteria isolates and used for their probable identification. These include; Gram's staining, motility, catalase test, oxidase test, gelatine hydrolysis, citrate utilization, indole production, methyl red test, acetylmeyhyl carbinol production (Voges Proskauer test), carbohydrate metabolism (hush and leifson's test), starch hydrolysis, acid-fast test, urease activity, nitrate test, acid and gas production from sugars (Holt et al., 1994). The identities of the three bacteria with strong growth on crude oil were further established by API techniques (API 20 NE for non-fastidious, non-enteric Gram negative rods and API 50 CHB/E for *Bacillus* and related general).

Hydrocarbon utilizers and bioaugmentation protocols

The isolation of hydrocarbon oil degraders was carried out by seeding the minimal salts agar medium with colonies isolated from the soil sample. This was then inverted on to a Petri dish cover containing filter paper soaked with each hydrocarbon for each isolate. The hydrocarbon served as the major carbon source. Incubation was done at 28°C for 48 to 72 h. Each colony was then picked with a sterile aluminium loop, emulsified in distilled water and 0.1 ml aliquots plated unto the minimal salts agar plates to obtain distinct colonies. The isolated strains were maintained on nutrient agar medium, incubated for 24 h and kept at 4°C.

The ability of these organisms to degrade crude oil and crude products was further tested by culturing in minimal salt broth with crude and crude oil respectively as sole carbon source. The Minimal Salt Broth (MSB) contain 2.134 g of Na_2HPO_4, 1.301 g of KH_2P0_4, 0.506 g of NHCl, 0.203 g of $MgSO_4$, 0.055 g of yeast extract, 1 ml of trace element. Each test-tube was filled with 9.0 ml minimal salt broth and 1% crude oil/products, then autoclaved at 121°C for 15 min. The isolates were then aseptically inoculated into the minimal salt broth and plugged with cotton wool to allow for aeration. These tubes were incubated at 30°C for 7 to 14 days with intermittent shaking to allow contact between the oil phase and the liquid phase, which contain the bacterial isolates. The amount of growth was observed comparatively with the control medium set-up, containing no bacterial inoculum. Observations and analyses of cultural surface growth, turbidity, pH, total viable count (TVC) and deposit/ residue were done and recorded (Amund et al., 1987).

Total viable count estimation

Total viable counts were determined by plating serially diluted samples from the broth culture containing crude oil on nutrient agar. One milliliter of each diluted samples was aseptically pipetted into sterile Petri dish which was in triplicates. Fifteen millilitres each of autoclaved molten nutrient agar was then added to the Petri dishes and left to cool to about 45°C. The Petri dishes were gently swirled

to spread the molten agar evenly on the plates. The agar was allowed to solidified and incubated at 30°C for 48 h. Total viable bacterial counts present in the samples were determined at days 0, 3, 6, 9, 12, 15, 18 and 21. The total viable bacteria were expressed as colony forming unit/l (cfu/l) (Nwachukwu et al., 2010).

Determination of oil concentration

The residual crude oil concentration in the soils samples were determined according to Vallejo et al. (2001), 8 g of the soil was extracted using 20 ml hexane in a flask tilted with a cap. The residual crude oil was analysed by 8200 auto sampler gas chromatograph equipped with a 50 m fused silica open tube capillary column internally coated with crosslinked methyl silicon and flame ionization detection. The degradation percentage was determined using the following formula according to Bento et al. (2005). Percentage degradation = {(Total crude oil in sample control - total crude oil in treated sample)/total crude oil in control}*100.

pH determination

A bench pH meter was used for this purpose. The meter was first standardized against standard buffer solutions of known pH values. The electrode of the meter was then washed with distilled water and then immersed in the sample contained in a beaker. The pH of the sample was then read on the pH meter scale and noted.

Plasmid extraction (curing of plasmid)

Twenty-four hours' culture of 1.5 ml was spinned for 1 min in a micro-centrifuge to pellet cells. The supernatant was gently decanted, leaving 50 to 100 µl together with cell pellet. 300 µl of TENS was added, and then mixed by inverting tubes 3 to 5 times until the mixture became sticky. Addition of 150 µl of 3.0 M sodium acetate pH 5.2 was done, and vortexed to mix completely, spanned for 5 min in micro-centrifuge to pellet cell debris and chromosomal DNA. Then supernatant was transferred into a fresh tube; mixed well with 900 µl of ice-cold absolute ethanol, spanned again for 10 min to pellet plasmid DNA (White pellet was observed). Supernatant was discarded and the pellet was rinsed twice with 1 ml of 70% ethanol and dried (TENS composition: Tris 25 mM, EDTA 10 mM, NaOH 0.1 N and SDS 0.5%). Plasmid-cured colonies were regenerated and further tested for biodegradative activities (Anna et al., 2008).

RESULTS

Physiochemical properties of soil from which the bacteria were isolated

The soil sample was low in all the parameters measured, probably because it was taken from the subsoil (Table 1).

Table 1. Physiochemical properties of soil sample.

Parameter	Soil
Texture	Loamy
pH	6.86
Moisture (vol. %)	12.90
Sodium (Na) (wt %)	0.0054
Potassium (K) (wt %)	0.0022
Magnesium (Mg) (wt %)	0.0395
Calcium (Ca) (wt %)	0.1820
Nitrogen (N) (wt %)	0.0248
Phosphorus (P) (wt %)	0.0011

Substrates specificity test

The ability of the organism to degrade hydrocarbons varied. Five of the bacterial isolates were hydrocarbon degraders and were further tested for substrate specificity. All the isotates were unable to grow on hexane. B1 and B2 had a luxuriant growth on n-dodecane and n-hexadecane. Only B1, B4 and B5 were able to grow on paraffin. B1 and B5 had poor growth on xylene. B1 and B3 had moderate/strong growth in phenol. B2, B4 and B5 had strong growth on crude oil. All the isolates had little growth on kerosene.

Residual oil concentration

The laboratory research carried out using crude oil as the sole source of carbon with bacteria isolated from soil samples (*Citrobacter koseri, Serratia ficaria* and *Bacillus coagulans*) resulted in higher degradation percentage as shown by the reduction in the peaks of the treated sample compare to the control. From the GC profiles, reduction in peaks and values for biomarkers, namely nC17/pristane and nC18/phytane ratio were much more pronounced in the "a" figures than the "b" figures. This was due to the absence of augmented bacteria in the soil samples.

The pH and growth curve

The pH of the crude oil enriched medium fell from 7.2 at day 0 to 7.0 at day 21; this was the same for the three isolates. The total viable cells went through the shortest lag phase of zero day in medium augmented with *B. coagulans* and the longest lag phase of six days in medium augmented with *C. koseri*. *B. coagulans* had the longest exponential phase of twelve days while *C. koseri* had the shortest exponential phase of three days. The stationary phase set in for the three isolates after day 12 of the study. Only *S. ficaria* with the shortest stationary phase shows no further minimum growth potential, thereby entering the death phase at day 18 of the study.

Degradation percentage of isolates

Table 5 shows that B4 (*B.* coagulans) was the best

degrader (95.11%) among the isolates from the soil sample used. B2 (*C. koseri*) and B5 (*S. ficaria*) were also able to degrade the crude oil maximally with a degradation percentage of 91.46 and 92.75 respectively in the laboratory.

Degradative dependency

Plasmid curing of the three isolates and thereafter their exposure to crude oil showed different degradative dependency. The degradative dependency abilities of *B. coagulans* and *C. koseri* were chromosomal mediated while *S.ficaria* was plasmid mediated.

DISCUSSION

In this study, the acidic pH value of the loamy soil sample could be interconnected with buildup of acidic metabolites and low mineral content of the soil (Ijah and Abioye, 2003). Phosphorous which is a limiting soil element and the exchangeable bases (Na^+, K^+, Ca^{2+} and Mg^{2+}) in the soil sample were low (Table 1). This could be that the geobiochemical activities in this soil using up these elements are more compared to those processes returning/accumulating them in the soil (DeBano and Klopatek, 1998; Abebe and Endalkachew, 2011). The low pH value might also be responsible for the poor presence of these bases in the soil sample in this study. Low pH (acidic) favours the abundance of exchangeable anions, but reduced cation, while high pH (basic) favours the abundance of exchangeable cations, but reduced anions in soils (Oyedele et al., 2008).

The bacteria isolates used in this work were from this soil sample (loamy soil) (Table 1) with no history of oil contamination. Crude oil degrader can be found in places other than crude oil polluted areas. All the isolates had different hydrocarbon degrading abilities (Table 2). Two of the isolates were Gram positive while the remaining three were Gram negative. The three isolates with the highest crude oil degradative potentials that were further screened (Tables 3 and 4) showed that *B. coagulans* was Gram positive while *C. koseri* and *S. ficaria* were Gram negative. Two-third of most petroleum hydrocarbon degraders are Gram negatives with one-third being Gram positive (Okerentugba and Ezeronye, 2003). *B. coagulans* and *S. ficaria* were better crude oil degraders than *C. koseri* (Table 5). Although, the three are facultative anaerobes which will help their adaptation in the anaerobic condition that might be created by the crude oil, *B. coagulans* and *S. ficaria* are spore former, which enhances their adaptation and degradation of crude oil (Nicholson et al., 2000; Forney et al., 2004; Ashlee et al., 2008). They were all rod-shaped. A lot of rod-shaped bacteria have also been implicated in hydrocarbon degradation (Okoh, 2003; Perfumo et al., 2007; Alfreda and Ekene, 2012).

Table 2. Substrate specificity test of isolates on different carbon sources.

Substrates	B1	B2	B3	B4	B5
Crude oil	+	++	-	+++	++
Xylene	+	-	-	-	+
Phenol	++	-	++	-	-
Engine oil	+	-	-	+	-
Diesel	-	-	+	+	-
Kerosene	+	+	+	+	+
Benzene	+	+	-	+	+
Cyclohexane	-	+	+	+	+
Paraffin	+	-	-	++	+
n-Decane	++	-	-	-	+
n-dodecane	+++	++	+	+	-
n-hexadecane	+++	+++	-	-	++
Hexane	-	-	-	-	-

-, No growth; +, poor growth; ++, moderate growth; +++, luxuriant growth.

Figures 1 to 5 show the gas chromatography (GC) profiles of the residual oil concentration (ROC). The peaks in the control sample after 21 days show no difference from day zero. On the contrast, there were pronounced reductions in peaks and in values of the samples inoculated with *B. coagulans, C. koseri* and *S. ficaria* after 21 days. Most importantly these chromatographic characteristics corresponded to criteria used to quantify petroleum degradation due to microbial activities (Wang et al., 1994; Yveline et al., 1997). It can be said that the crude oil was metabolized/degraded by these bacteria. Also, the initial total viable count (TVC) (Figures 6 to 8) in each culture was low which could be attributed to the initial possible toxicity of the hydrocarbon on the bacteria cells alongside the gradual adaption of the bacteria to the crude oil presence (Head et al., 2006; Yakimov et al., 2007; Maduka and Okpokwasili, 2016). The impact of hydrocarbons on the bacteria may not be directly related to their toxicity. It could be the destruction of inorganic nutrient sources that are essential for microbial growth and catabolic activities. Hydrocarbons have ability to react and form complexes with nitrates, sulphates and phosphates, thus making them unavailable to the soil organisms (Adams and Jackson, 1996). This adaption process corresponds to the lag phase, which was shortest for *B. coagulans*. It must be mentioned that *Bacillus* spp. have been isolated from different environment including extreme environment, implicated in many hydrocarbon biodegradation researches and also known to be versatile in their substrate usage (Sepahi et al., 2008; Olukunle and Boboye, 2013: Lateef and Oluwafemi, 2014; Kulkarni and Kaliwal, 2015; Maduka and Okpokwasili, 2016). These are the reasons why *B. coagulans* had the shortest adjusting period (lag phase) which resulted to highest degradation percentage

Table 3. Biochemical characteristic of B2 and B5 using API 20E.

Tests	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
B2	+	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	-
B5	+	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-

B2; *Citrobacter koseri;* B5; *Serretia ficaria.* **ONPG**, Ortho nitrophenyl,ßD,galactopyranosidase; **ADH**, arginine dihydrolase; **LDC**, lysine decarboxylase; **ODC**, ornithine decarboxylase; **CIT**, citrate utilization (trisodium citrate); **H_2S** production; **URE**, urease; **TDA**, tryptophane deaminase; **IND**, indole production; **VP**, Voges Proskauer (sodium pyruvate acetoin production); **GLU**, glucose; **INO**, inositol; **SOR**, sorbitol; **RHA**, rhamnose; **SAC**, saccharose; **MEL**, melibiose; **AMY**, amygdalin; **ARA**, arabinose; **OX**, oxidase.

Table 4. Biochemical characteristic of B4 using API 50 CHB/E.

Tests	GLY	ERY	DARA	LARA	RIB	DXTL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB
B4	+	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+
ESC	**SAL**	**CEL**	**MAL**	**LAC**	**MEL**	**SAC**	**TRE**	**INU**	**MIZ**	**RAE**	**AMD**	**GLYG**	**XLT**	**GEN**	**TUR**	**LYX**	**TAG**	**DEUC**	**LEUC**	**DARL**	**LARL**	**GNT**	**2KG**	**5KG**
+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-	+	-	+	-	-

B4; *Bacillus coagulans.* **MDM**, Methyldmannoside; **NAG**, N-acetylglucosamine; **ARB**, arbutin; **SAL**, salicin; **CEL**, cellobiose; **MAL**, maltose; **LAC**, lactose; **TRE**, trehalose; **INU**, inulin; **GLY**, glycogen; **GEN**, gentiobiose; **TUR**, turanose; **LYX**, lyxose; **TAG**, tagatose; **DARA**, D-arabitol; **LARA**, L- arabitol; **ERY**, erythritol; **RIB**, ribose; **LXYL**, L-xylose; **ADO**, adonitol; **GAL**, galactose; **FRU**, fructose; **DUL**, dulcitol; **2KG**,2,Ketogluconate; **5KG**,5,Ketogluconate.

Table 5. Percentage of crude oil degraded.

Lab code	Oil concentration at day 0 (mg/ml)	Oil concentration at day 21 (mg/ml)	% Degraded
B4	3728.98	182.33	95.11
B5	3728.98	311.58	92.75
B2	3728.98	270.27	91.46

compared to the other two bacteria (*C. koseri and S. ficaria*). It also had the longest exponential phase of twelve days, with *C.koseri and S. ficaria* having the exponential phase of six and nine days respectively. The above reasons, also must have led to *B. coagulans* having the highest degradation percentage (95.11%). High growth (increased biomass) at the exponential phase resulted to the utilization of the crude oil (Okpokwasili and Nweke, 2006; Nour et al., 2014). *C. koseri* and *S. ficaria* had growth reduction after day 12 (although later increased after day fifteen), which is the onset of the stationary phase. This definitely affected their degradation percentages (Table 5).

The relationship between pH and biodegradation was also observed. The pH of the medium was observed to drop from 7.2 to 7.0 for the different augmented bacteria. This is probably because degradation of the hydrocarbon had resulted in the release of acidic substances (organic acids and other metabolic products) which reduces the pH of the medium (Sepahi et al., 2008; Maduka and Okpokwasili, 2016). The pH changed a little within the 21 days' period of the research, the acidic substances must have been released minimally. Therefore, the pH remained in optimal level, conducive for these bacteria activities.

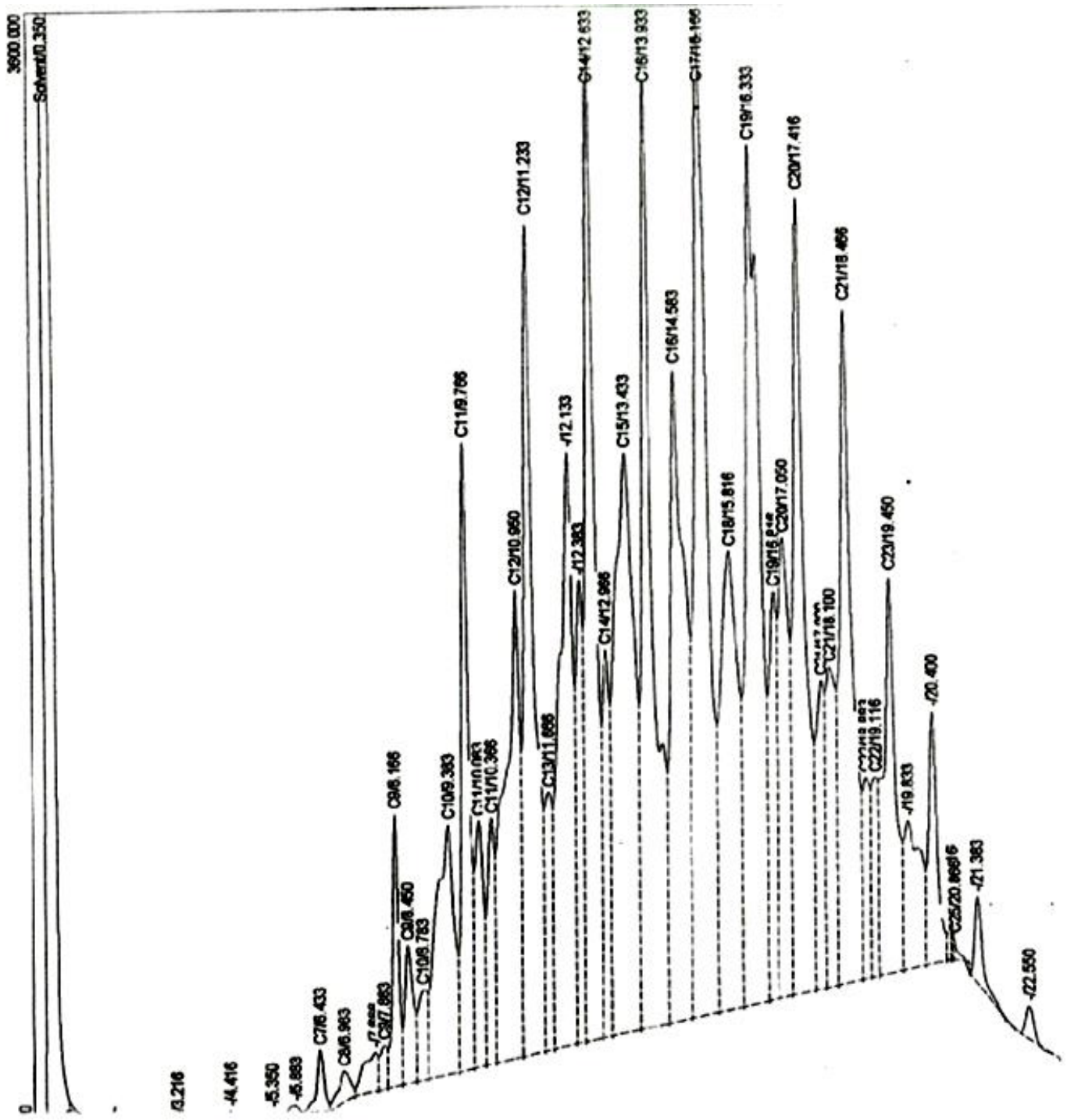

Figure 1. Gas chromatogram of the residual oil concentration present in the control sample at day 0.

The influence of plasmid in crude oil degradation by the three bacteria was evaluated. The three bacteria possess plasmid. They were then cured of the plasmid and the effect of plasmid was established when the isolates were re-introduced into crude oil containing culture. Only two were able to degrade the crude oil after curing (Table 6). This underscored the role of plasmid in their crude oil degradation activities. It can be concluded that the genetic factor responsible for crude oil degradation in *B. coagulans* and *C. koresi* were chromosomal mediated while that of *S. ficaria* was plasmid mediated. Further research can reveal the crude oil degrading associated

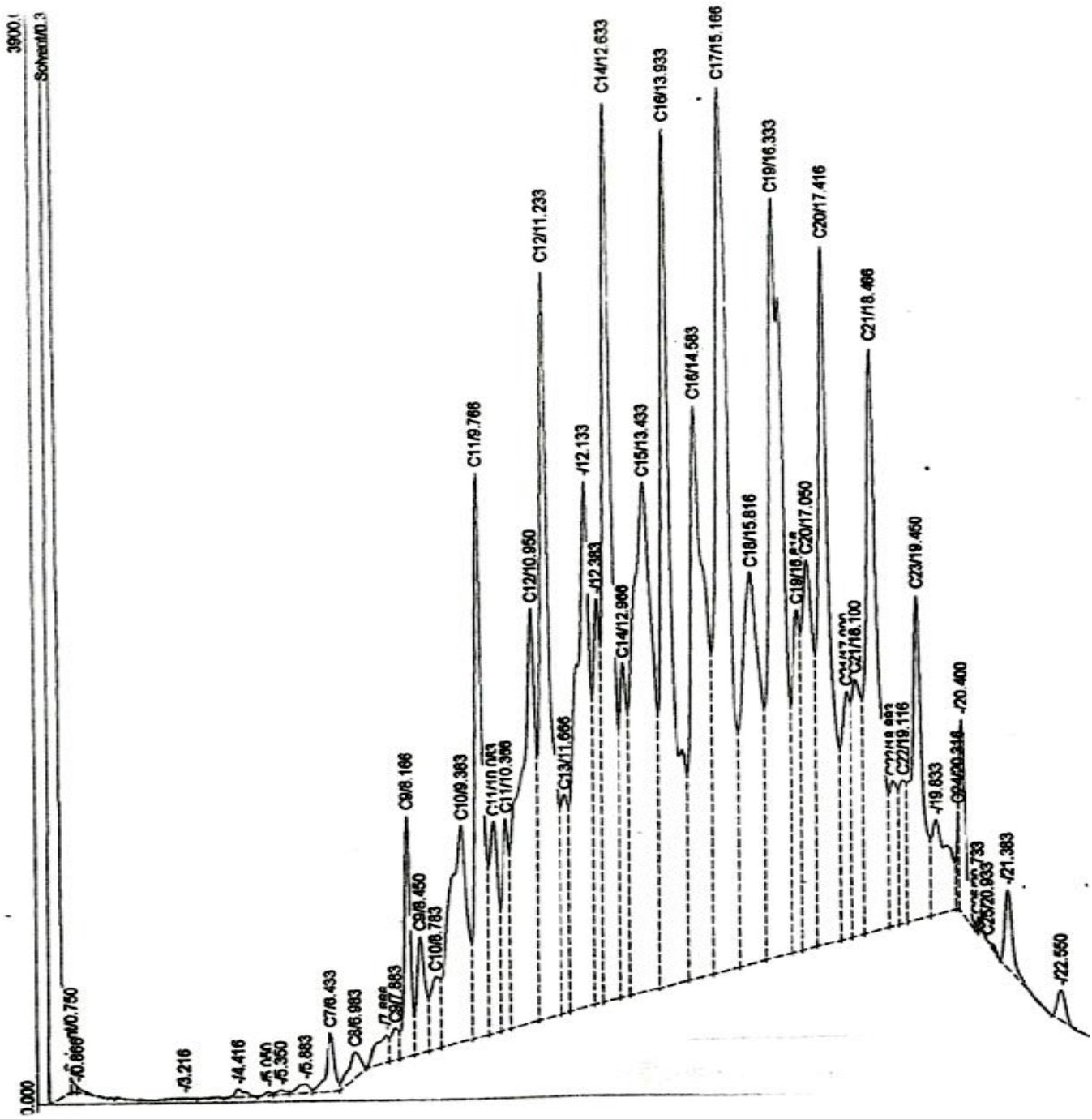

Figure 2. Gas chromatogram of the residual oil concentration present in the control sample day 21.

gene in these bacteria which could be as one of the biomarkers in identifying crude oil degrading bacteria. This will improve strain selection for bonny light crude oil degradation, which will be similar to bamA gene. Gene bamA encodes the enzyme 6-oxocyclohex-1-ene-1-carbonyl- CoA hydrolase that can degrade aromatic hydrocarbon, which was proposed to be used as a biomarker for the detection of aromatic compound degrading microorganisms (Kuntze et al., 2008; Kuntze et al., 2011; Ruan et al., 2016; Xiaoyun et al., 2016). In addition the chemotaxis response of some bacteria towards pollutants depends on some gene function which could also be associated to presence of plasmid in a bacteria cell. The inability of *S. ficaria* to degrade crude oil after curing was due to the removal of its chemotaxis dependence molecular unit, needed to respond to the

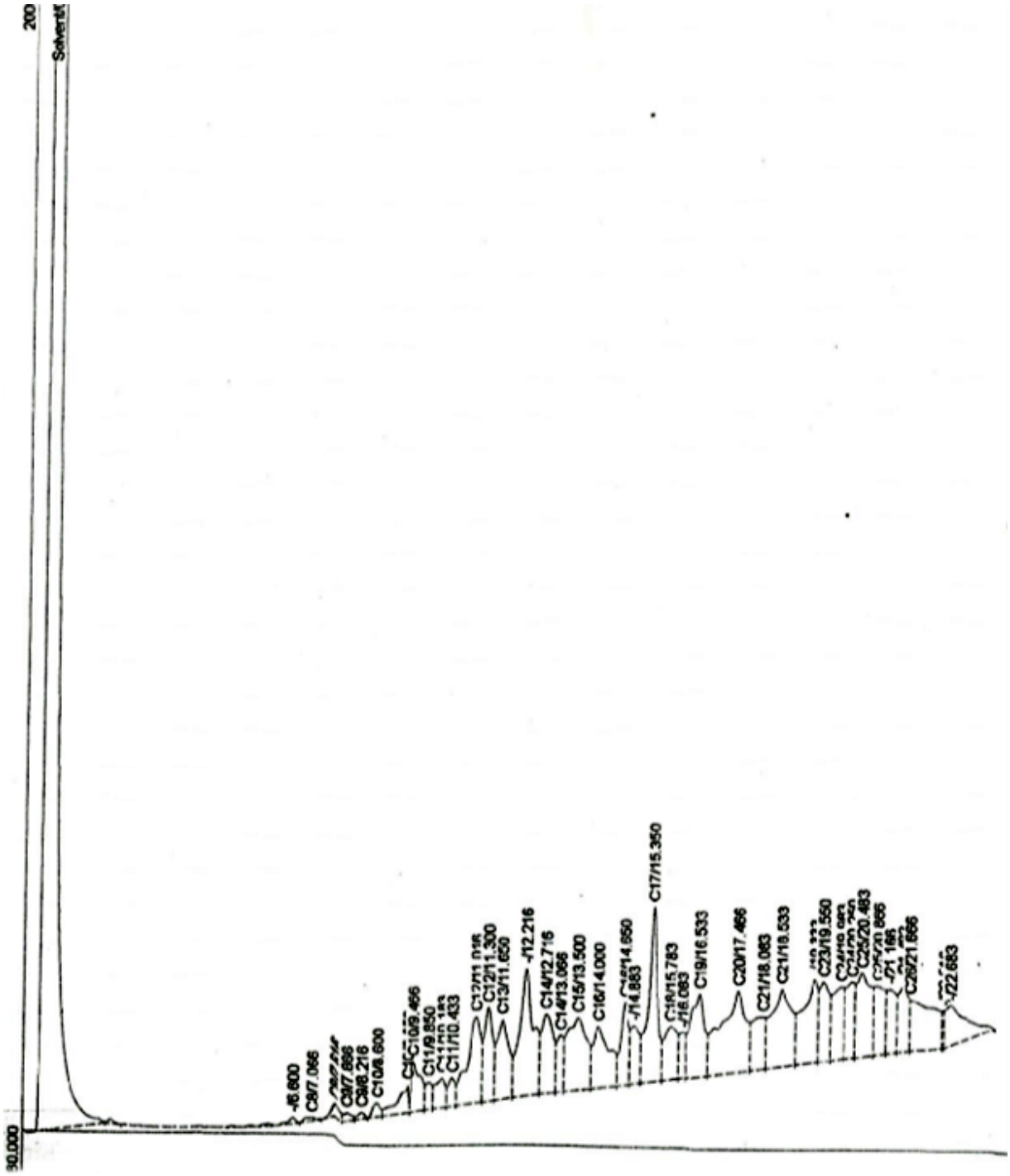

Figure 3. Gas chromatogram of the residual oil concentration in sample inoculated with *Citrobacter koseri* after 21 days.

crude oil presence and its subsequent degradation (Gunjan and Rakesh, 2002). Plasmid mediated degradation of substances by some bacteria have also been documented. Plasmid-mediated degradation of dimethoate was observed in *Pseudomonas aeruginosa* and *Bacillus licheniformis;* Chlorpyrifos by *Micrococcus*, *Enterobacter* and *Pseudomonas* with plasmid borne genes for its degradation (Guha et al., 1997; Singh et al., 2003; Bhagobaty and Malik, 2008; Kulkarni and Kaliwal, 2015).

Conclusion

Bacteria degradative dependency ability can be grouped

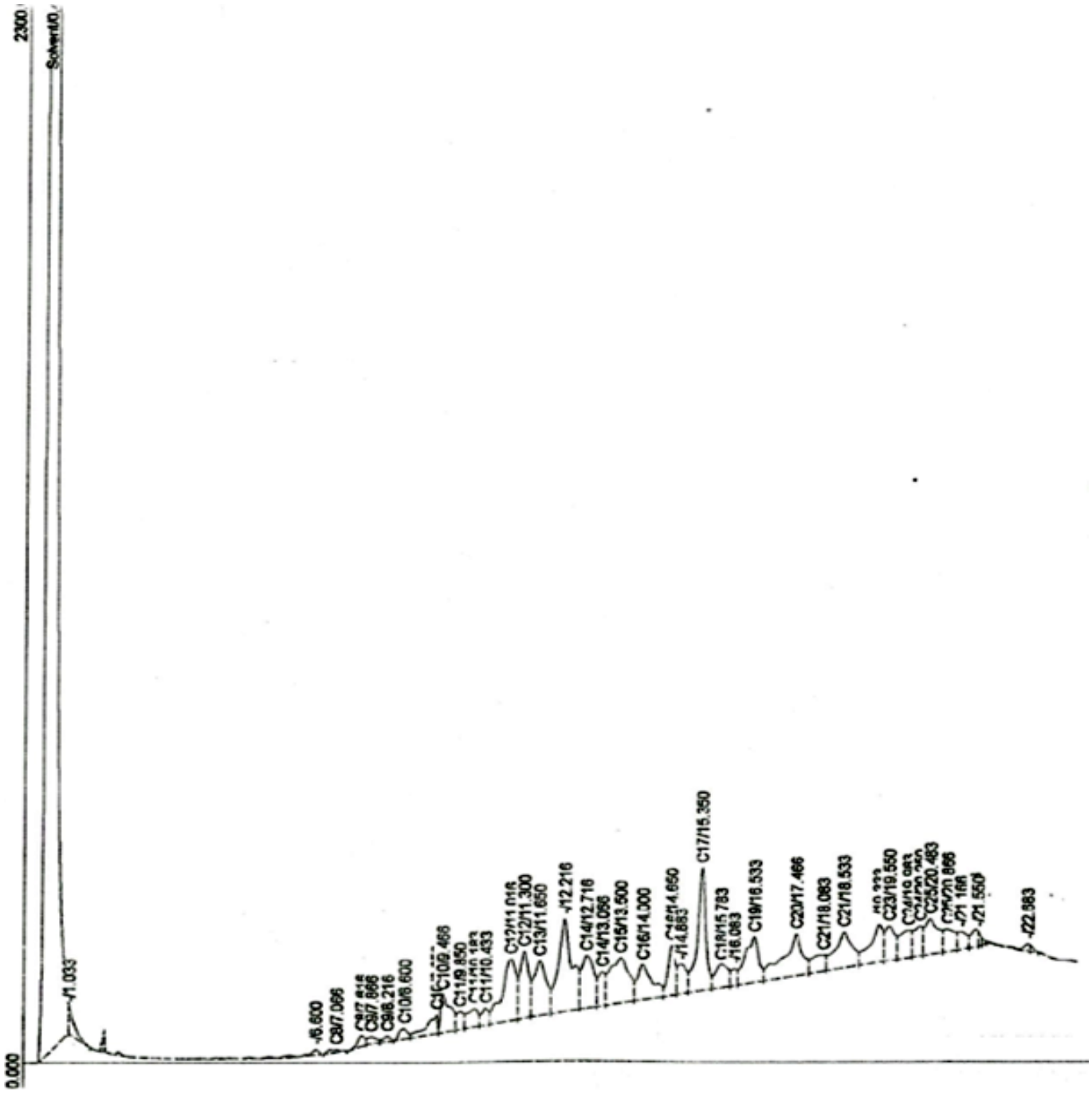

Figure 4. Gas chromatogram of the residual oil concentration present in sample inoculated with *Bacillus coagulans* after 21 days.

into chromosomal and plasmid mediation. Bacterial species isolated in this work have shown to be excellent degraders of crude oil. These bacteria could show greater biodegradating ability with further studies, since their degradative dependency (chromosome and plasmid) have been established in this study. The plasmid of *S. ficaria* can also be used in recombinant DNA technology to develop bacteria cell with potential to degrade crude oil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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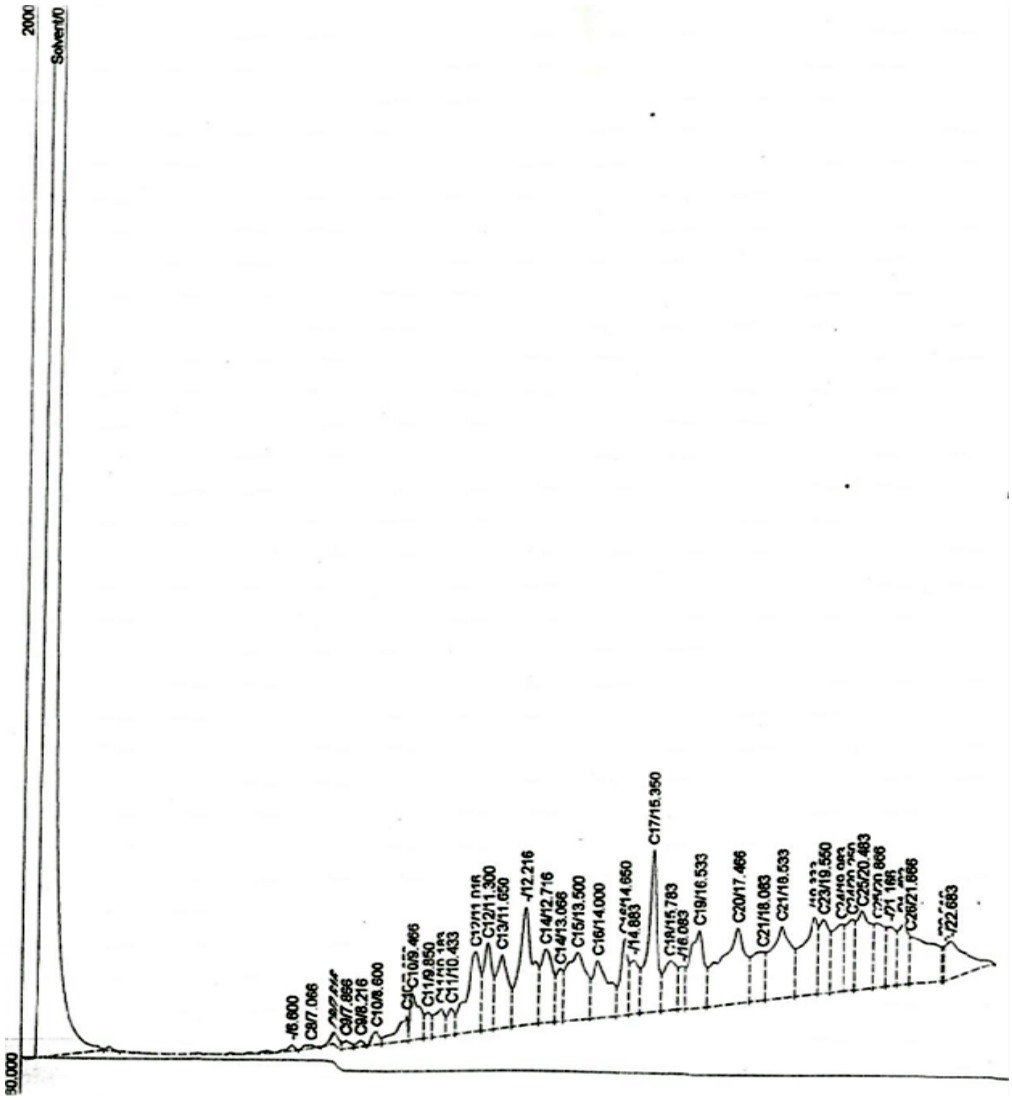

Figure 5. Gas chromatogram of the residual oil concentration present in sample inoculated with *Serretia ficaria* after 21 days.

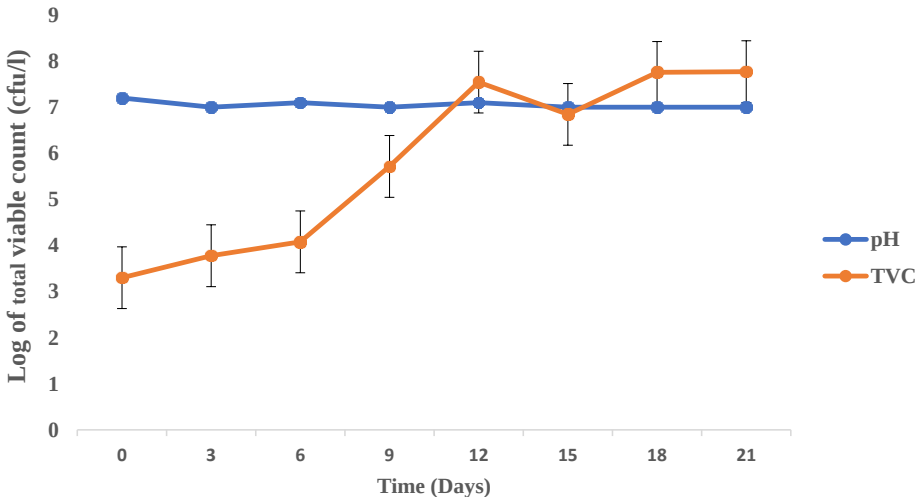

Figure 6. Growth curve of *Citrobacter koseri* on crude oil as sole carbon source.

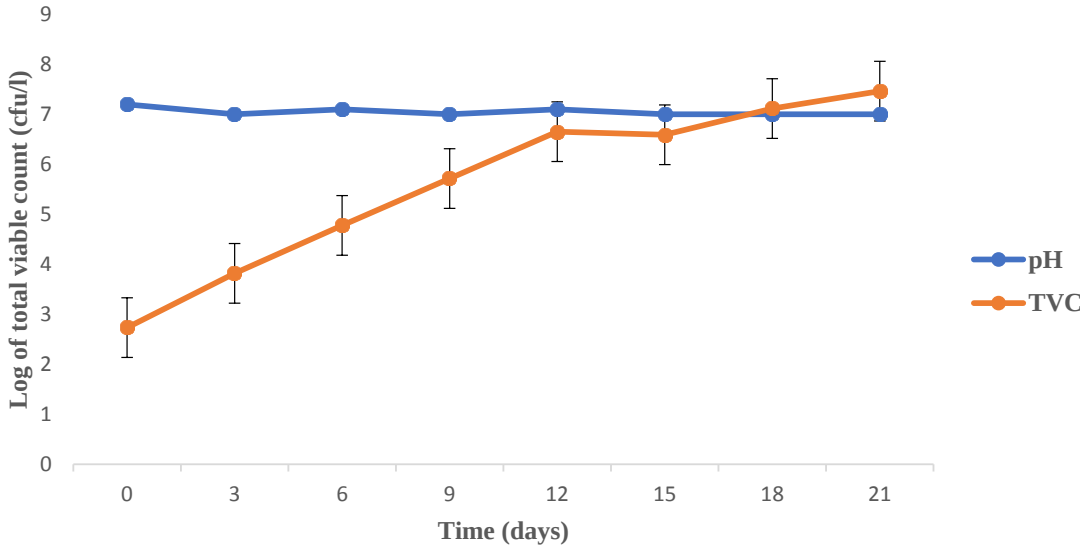

Figure 7. Growth curve of *Bacillus coagulans* on crude oil as sole carbon source.

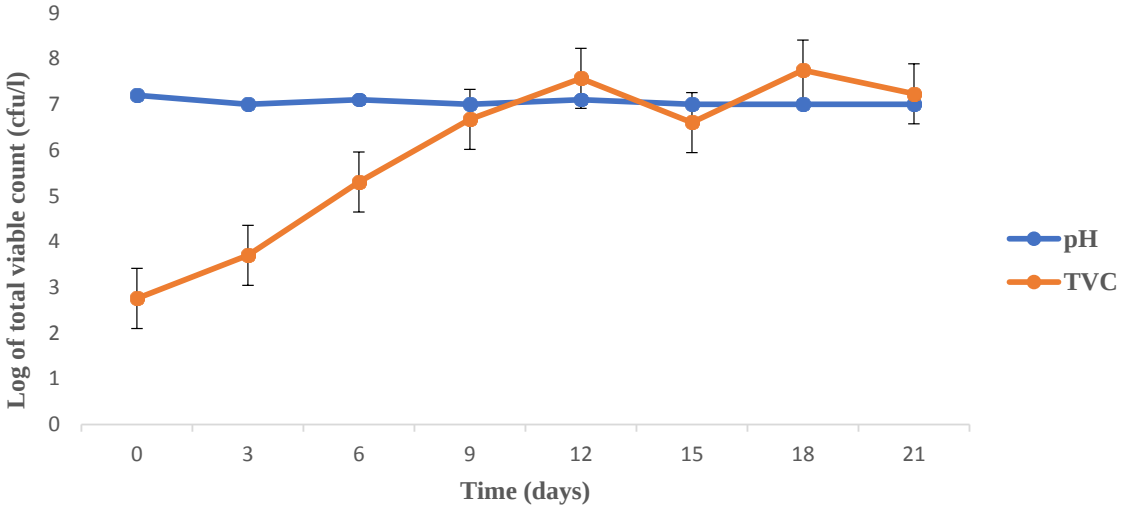

Figure 8. Growth curve of *Serratia ficaria* on crude oil as sole carbon source.

Table 6. Crude oil degradation after plasmid curing.

Organisms	Before curing	After curing
C. koseri	+	+
B. coagulans	+	+
S. ficaria	+	-

+ = ability to degrade; - = lose the ability to degrade.

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