



***Microbacterium lacticum*; a Lysine Producing Bacterium Isolated from Oil-contaminated Soil in South-East Nigeria**

C. C. Ezemba^{1*}, C. C. Ekwealor¹, I. A. Ekwealor¹, C. A. Ozokpo¹,
C. E. Chukwujekwu², V. N. Anakwenze¹, E. J. Archibong¹ and G. C. Anaukwu¹

¹Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, P.M.B. 5025, Awka, Anambra State, Nigeria.

²Department of Microbiology, University of Benin, Edo State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author CCE designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author CAO, CEC, VNA, EJA and GCA managed literature searches. Authors CCE and IAE managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To screen for lysine production using hydrocarbon utilizing bacteria isolated from oil-contaminated soil.

Study Design: Study of the isolation and fermentation process in shake flask culture.

Place and Duration of Study: Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria between 2009 to 2010.

Methodology: Collection of Samples: Enrichment of hydrocarbon- degrading bacteria was done in the basal medium. The carbon source consisted of 0.5 ml of the hydrocarbons added to a sterile filter paper secured in the lids of the Petri dishes. The isolates were screened for lysine production.

*Corresponding author: E-mail: constancechinyere790@yahoo.com;

Results: Sixteen of the one hundred and forty isolates were found to produce lysine on solid agar medium. The strains were tested for lysine production in their broth after 3 days at 30°C. Among the different hydrocarbons tested groundnut oil and motor oil were found to be suitable. Active strain designated G₁, which produced the highest lysine yield of 1.44 mg/ml under submerged condition was characterized. The gram-positive, irregular, slender rod, utilizing citrate, urease, tyrosine and reduces nitrate was identified as *Microbacterium lacticum*

Conclusion: We have isolated many strains of hydrocarbon utilizing microorganisms, in order to examine if the reasonable amounts of lysine of economic value can be produced by those microorganisms from hydrocarbons such as kerosene, gasoline, motor oil, spent oil, crude oil, which are considered to be more economical and readily available carbon sources than carbohydrates. Lysine producing bacteria can be isolated from Nigeria soil and improving cultural conditions of hydrocarbon utilizers in submerged medium accumulated lysine.

Keywords: Lysine; *Microbacterium lacticum*; culture medium; fermentation; soil.

1. INTRODUCTION

Out of the twenty naturally occurring amino acids, L-Lysine is one of the nine essential and commercially important amino acids, found in naturally occurring proteins of all living organisms [1]. Its major commercial form is L-lysine Monohydrochloride (L-lysine-HCL). Good sources of lysine are foods rich in protein like meats, cheese, certain fish, nuts, eggs, soyabeans. L-Lysine is nutritionally important to man and animals and can be used to supplement food and food materials especially cereal products to improve protein quality [2]. L-Lysine is utilized in human medicine, in cosmetics, in the pharmaceutical industry, particularly as ingredients of amino acid infusion and as precursor for industrial chemicals [3].

In the recent times, a lot of research efforts have been geared towards the production of amino acids by fermentation methods. Many of these processes seem to be the most economical and practicable means of producing optically active and more readily utilizable amino acids [4]. Also interest in the microbial biodegradation of pollutant has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments [5,6].

This research was therefore carried out to isolate and screen lysine producing bacteria utilizing hydrocarbon from different soils in south-eastern part of Nigeria.

2. MATERIALS AND METHODS

2.1 Microorganisms

The microorganisms used for this study were *Escherichia coli* DSM 5210 and *Escherichia coli*

DSM 1099 obtained from Deutsche sammlung von Mikroorganismen und Zeellkulturen GmbH, Germany. It was reconstituted on broth and maintained on Nutrient agar (oxid) slant at 4°C

2.2 Collection of Samples

2.2.1 Soil

Oil contaminated soil samples were collected and kept in a sterilized sterile screw capped bottles from the following locations in South-eastern part of Nigeria, at the soil depth of 2-8 cm.

- 1) Nigerian National Petroleum Corporation (NNPC) refinery at Warri, Delta state.
- 2) Nigerian National Petroleum Corporation (NNPC) refinery Port Harcourt, Rivers State.
- 3) Mechanical workshop, Heritage Street, Omagba Onitsha, Anambra state.
- 4) Mechanical workshop, Awka road, Onitsha Anambra state.
- 5) Refuse dump sites, Awka road, Onitsha Anambra state.
- 6) Forest situated at Mgbudu Ichida, Anambra state.

2.2.2 Hydrocarbons

Kerosene, gasoline, motor oil, were obtained from Liquid gold filling station, along Enugu-Onitsha express road and spent oil from a mechanic workshops at Awka road, in Onitsha, Anambra State. Crude oil was obtained from (NNPC) refineries in Warri and Port-Harcourt.

2.3 Isolation and Screening Hydrocarbon Utilizers

Enrichment of hydrocarbon- degrading bacteria was done in the basal medium (Ward and Brock, [7] consisting of: NaCl, 0.4g; NH₄Cl, 0.5g; MgSO₄.7H₂O, 0.5 g; KH₂PO₄, 0.05 g, distilled H₂O, 1 L and NaHPO₄.7H₂O, 0.05 g. Two grams of soil sample was serially diluted in ten- folds in sterile distilled water. One drop of hydrocarbon and 0.1 ml of 10⁻⁵ dilutions of soil sample were introduced into 10ml of sterilized basal medium. The tube was incubated at 30°C for 7 days. Pure cultures of hydrocarbon-utilizing bacteria were isolated from the enrichment culture by streaking onto sterile basal medium to which 2% agar was added. The carbon source consisted of 0.5ml of the hydrocarbons added to a sterile filter paper secured in the lids of 70 by 15 mm-diameter sterile Petri dishes under aseptic condition. The dishes was then inverted and incubated at 30°C for 4 days. The pure isolate were transferred onto Nutrient agar (Oxoid) slants and stored at 4°C for further studies.

2.4 Preliminary Screening of Isolates for Lysine Production on Solid Medium

The isolates were screened for lysine production by the method described by Hallsall [8]. Sterilized plates of the minimal agar medium containing: KH₂PO₄, 1.36 g; (NH₄)₂SO₄, 2.0 g; MgSO₄.7H₂O, 0.2 g; CaCl₂, 0.01 g; FeSO₄.7H₂O, 0.5 mg; Glucose, 2.0 g; Agar, 12 g; distilled H₂O, 1L, pH adjusted to 7.2 with 1N of NaOH and sterilized at 121°C for 15 minutes. The minimal agar medium seeded with 24h broth culture of lysine auxotrophs, *Escherichia coli* (DSM 1099 and DSM 5210) was, spread inoculated with each soil isolate. After 48-72h incubation at 30°C, the plates were examined for growth of the auxotroph. A total of hundred and forty hydrocarbon-utilizers were similarly screened, and the Gram-stain reaction of the lysine-producing bacteria was recorded.

2.5 Production of Lysine in Shake Flask Fermentation

2.5.1. Seed medium

The medium for seed inoculum consists of: Peptone, 10.0g; Yeast extract, 10.0 g; NaCl -5.0 g; distilled H₂O -1litre, pH adjusted to 7.2 with 1N of NaOH and sterilized at 121°C for 15 min. Two loopfuls of the lysine-producing isolate were inoculated into a test tube containing 5ml of the

seed medium. The test tube was incubated for 16-18 h on a shaker (120 rpm) at 30°C.

2.5.2. Fermentation

The basal medium for fermentation is composed of: KH₂PO₄, 1.0 g; MgSO₄.7H₂O, 0.4 g; MnSO₄.H₂O, 2.0 mg; FeSO₄.7H₂O, 2.0 mg; CaCO₃, 50.0 g; Glucose, 20.0 g; (NH₄)₂SO₄, 10.0 g; distilled H₂O, 1 L, pH adjusted to 7.2 with 1N NaOH and sterilized at 115°C for 10 min. A 2-ml (ca 1.56 x 10⁸ cells/ml) of the seed inoculum was used to inoculate duplicate Erlenmeyer flask containing 100 ml of fermentation medium. Four uninoculated flasks served as control. After 72h incubation on Gesellschaft (D3006) rotary shaker at 160 rpm at 30°C, growth and lysine produced were determined from the broth culture. Lysine assay were carried out in duplicates and uninoculated flask served as control.

2.6 Determination of Growth

Growth of the isolate was determined turbidimetrically from the culture broth using JENWAY Spectrophotometer (Model 6405 uv/vis) at 660 nm.

2.7 L-Lysine Assay

Quantitative estimation of L-lysine in the supernatant was carried out by acid ninhydrin method of Chinard [9]. A 5ml volume of the culture broth was centrifuged at 5000 × g for 20 min and the cell-free supernatant was assayed for L-lysine. One ml of glacial acetic acid was added to 1ml of the supernatant in a test tube followed by the addition of 1ml of a reagent solution which contains an acid mixture of 0.4ml of 6 M orthophosphoric acid, 0.6 ml of glacial acetic acid and 25 mg of ninhydrin per millilitre of the acid mixture. The blank contains 1ml of glacial acetic acid, 1ml of the acid mixture without ninhydrin and 1ml of the supernatant. Both test tubes were capped and the content mixed properly before heating at 100°C in a water bath for 1 h. The test tubes were cooled rapidly under tap water and 2 ml of glacial acetic acid added to each tube to give a final volume of 5 ml. The optical density of the reacting mixture was read against the blank at 515 nm in a spectrophotometer. The bacteria isolates with the highest production of lysine was selected for further studies. The amount of lysine produced was extrapolated from a lysine standard curve and lysine concentrations were estimated. The standard lysine curve was obtained by plotting the values of optical densities against the

concentrations (0.1 mg/ml to 0.9 mg/ml) of standard lysine solutions.

2.8 Characterization of Isolate

Biochemical tests performed for the characterization of the isolate include: Growth on Nutrient agar, Growth on Yeast Extract-Peptone Glucose Agar, Growth on MacConkey agar, Gram staining, Spore staining. Catalase reaction, Motility test, Citrate Nitrate reduction test, Urease test, Indole test, Methyl Red (MR) test, Voges Proskauer test, Triple sugar iron test, Starch hydrolysis Sodium chloride tolerance, Oxidase test, L-Tyrosine utilization, Tween 80 Phenylalanine test and Sugar fermentation. The following sugars were tested: Glucose Lactose and Mannitol.

3. RESULTS AND DISCUSSION

The search for cheaper lysine production alternative has guided our study. The ability of microorganisms to utilize hydrocarbons as a sole carbon source has been recognized for many years. Use of selective media containing hydrocarbons were used for the production of lysine-producing bacteria. From the nature of screening for lysine on solid medium and in shake flask fermentation, *Microbacterium lacticum* (Table 1) was chosen as active lysine producer and was characterized. As presented in Fig. 1, all bacteria were able to degrade the hydrocarbons tested. Although kerosene and spent oil were the most highly occurring hydrocarbon utilized as the carbon and energy source during enrichment process, other different compounds could also be utilized. The inherent capacity of these bacterial organisms to assimilate petroleum hydrocarbon and its products is supported by the work of Okpokwasili and James [10]. These workers isolated *Nocardia alkanoglutinosa* from soil, capable of utilizing various carbon sources like kerosene, crude oil, animal & vegetable fat and fatty acids. Also Guzik et al. [11] and Gutierrez et al. [12] isolated a Gram –negative bacterium identified as *Stenotrophomonas maltophila* and *Porticoccus hydrocarbonoclasticus* by enrichment of the medium with different aromatic hydrocarbons substrates as sole carbon and energy source.

In the search for microorganisms that are hydrocarbon utilizers, both Gram positive and Gram negative were isolated (Table 1). The ability of these bacteria to be predominantly

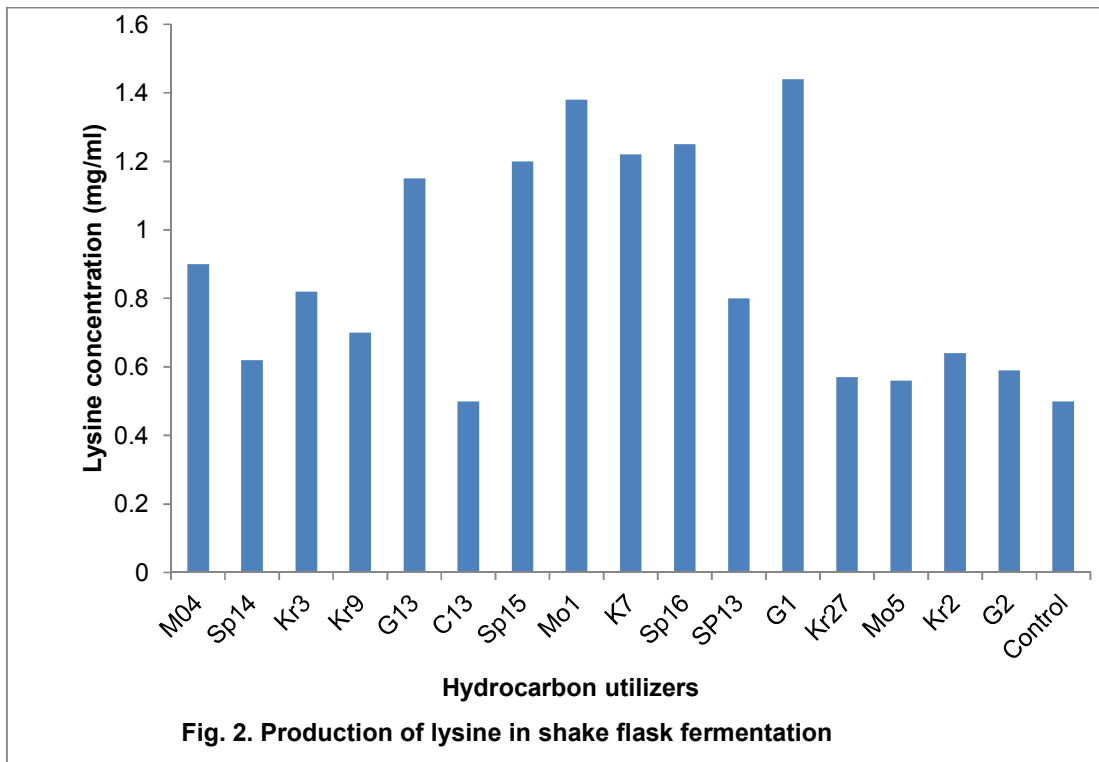
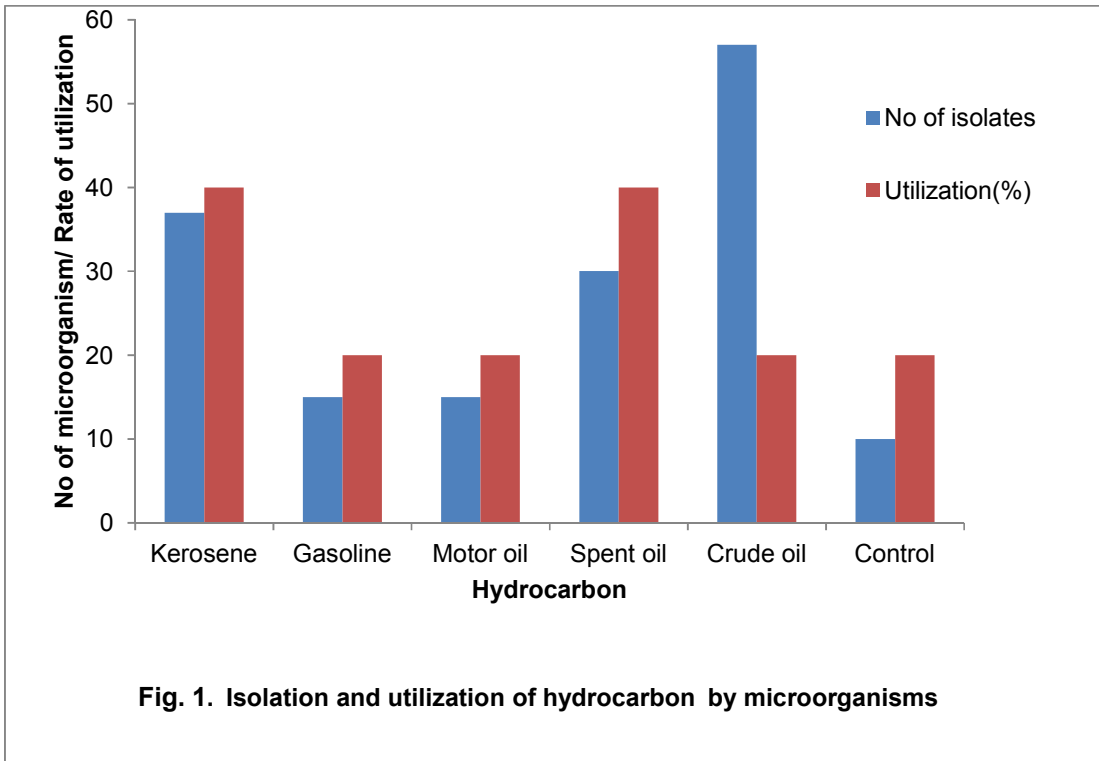
Gram-negative is contrary to Austin et al. [13] who isolated predominantly Gram-negative hydrocarbon utilizing bacteria from soil and aquatic environment.

The production of lysine by this hydrocarbon utilizers in Fig. 2, agrees with the work of Sen and Chatterjee [14] who isolated *Arthrobacter globiformis* from Burdwan (Indian) soil that accumulated 3.4 g/l of L-lysine and was able to utilize hydrocarbon (gas oil) as carbon source. They were also capable of accumulating L-lysine in purely synthetic medium. The ability of these bacteria to utilize hydrocarbon as their carbon source and to also produce lysine is in line with the work of [14-16]. Ekwealor and Obeta [17] also were able to isolate lysine producer *Bacillus megaterium* sp 14 from Nigerian soil. The lysine production using various hydrocarbons indicates that groundnut oil, motor oil and spent oil gave a high yield of 1.44 mg/ml, 1.38 mg/ml, and 1.20 mg/ml respectively. This finding is in contrast with the work of Wantanabe et al. [18] reported a high lysine yield when n-paraffins containing carbon preferably 13-18 atom or kerosene was added to the fermentation medium for Genus *Pseudomonas* and *Achromobacter* (Fig. 2). Yamada et al. [19], also observed an improved lysine production by different strains of hydrocarbon utilizing organisms when mixtures of hydrocarbon such as kerosene, ligroin and liquid paraffin was the carbon source in the culture medium.

Table 1. Preliminary screening of isolates for lysine production on solid medium

Isolate designation	Gram-stain reaction
Mo ₄	Gram positive cocci
Sp ₁₄	Gram positive cocci
Kr ₃	Gram positive rods
Kr ₉	Gram negative cocci
G ₁₃	Gram positive rods
C ₁₃	Gram negative rods
Sp ₁₅	Gram negative cocci
Mo ₁	Gram positive rods
K ₇	Gram negative rods
Sp ₁₆	Gram negative rods
Sp ₁₃	Gram positive cocci
G ₁	Gram positive rods
Kr ₂₇	Gram positive rods
Mo ₅	Gram positive cocci
Kr ₂	Gram positive rods
G ₂	Gram positive rods

KEY →Sp→Spent oil; Mo→Motor oil;
Kr→Kerosene;G→Gasoline; C→Crude oil



An active strain designated as G₁ was morphologically, physiologically and biochemically characterized using standard techniques as summarized in Table 3. A Gram positive rod and good growth was observed on Nutrient Agar, Yeast Extract-peptone Glucose Agar and MacConkey Agar. Both nitrate and citrate were reduced, but methyl red and Voges-Proskauer was not utilized by the isolate. G₁ utilized maltose, glucose and fructose while others were not utilized. Indole, starch hydrolysis, and L-tyrosine were positive while oxidase, and phenylalanine were negative. Based on the characteristic features and with reference to Buchanan and Gibbon [20], Bergeys's manual of determinative bacteriology, it was identified as *Microbacterium lacticum*.

The methodology was able to isolate lysine producing bacteria from oil contaminated soil using various hydrocarbons. This result shows that most bacterial species are capable of utilizing hydrocarbon, and also produce amino acids.

Microbacterium lacticum produced 1.44 mg/ml after 72 hours fermentation period. Statistical analysis of the result as shown in Table 2, shows that there is significant difference between various hydrocarbon and lysine concentration. This reports agrees with the works of [14-16,19,20], they reported lysine production by hydrocarbon utilizers.

Table 2. Statistical analysis of production of lysine in a shake flask fermentation

Isolate no.	Lysine (mg/ml)	Standard deviation
Mo4	0.90	±0.02
Sp14	0.62	0.06
Kr3	0.80	0.20
Kr9	0.70	0.04
G13	1.15	0.04
C13	0.50	0.06
Sp15	1.20	0.02
Mo1	1.38	0.03
K7	1.22	0.02
Sp16	1.25	0.03
Sp13	0.80	0.04
G1	1.44	0.01
Kr27	0.57	0.05
M05	0.56	0.04
Kr2	0.64	0.03
G2	0.59	0.06
Control	0.50	0.06

KEY →Sp→Spent oil; Mo→Motor oil; Kr→Kerosene; G→Gasoline; C→Crude oil; X: value represent the mean (± i.e. plus or minus) standard deviation of duplicate determinations

Table 3. Morphological, physiological and biochemical properties of Isolate G₁

Test	Characteristics features
Nutrient Agar	Yellow-white creamy colonies with wavy margins.
Yeast Extract-Peptone Glucose Agar	Colonies are opaque and glistening with yellowish pigmentation
Growth On MacConkey agar	Positive.
Gram stain	Positive with irregular slender rods arranged singly
Spore stain	-ve
Catalase	+ve
Motility	+ve
Citrate	+ve
Nitrate reduction	+ve
Urease	+ve
Indole	+ve
MR	-ve
VP	-ve
Triple sugar iron	BUTT→Acid (yellow) Slope→Alkaline (red) Gas → -ve
Starch hydrolysis	+ve
Sodium chloride	>9 < 10
Oxidase	-ve
L-Tyrosine	+ve
Tween 80	+ve
Phenylalanine	-ve
Fermentation sugars:	
Glucose	±
Lactose	-ve
Mannitol	-ve
Galactose	-ve
L-Arabinose	-ve
Maltose	+ve
Sucrose	-ve
Fructose	±
Dulcitol	-ve
D-xylose	-ve
Probable organism	<i>Microbacterium lacticum</i>

KEY => + = Positive; - = Negative; ± = Weakly positive

4. CONCLUSION

In this study, for the first time the isolation and characterization of Gram-negative *Microbacterium lacticum*, designated as G₁, which exhibits activities of producing lysine while

growing in the presence of hydrocarbon, has been reported. And it is very likely that by improving the strains of these bacterial species, high lysine production will be obtained and when these microorganisms are produced in large scale, they would be used for environmental remediation, hence restoring our land and water for safe use. Submerged fermentation process of lysine production when developed will reduce importation of lysine in Nigeria and make it more readily available.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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