Characterization of Biopolymers and Biosurfactant for Microbial Enhanced Oil Recovery in Niger Delta Reservoirs

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Abstract— Microbial EOR is a cost effective, environmental friendly technique used in the oil industry to increase recovery through the inoculation of microorganisms in a reservoir to extract residual oil by different mechanisms from microbial metabolites. For this study, Bacillus polymyxa (G2) and Pseudomonas aeruginosa (G1) were isolated, characterized and identified in the laboratory among other microbes using brackish water. This was based on the cultural morphology, microscopic examination and other biochemical tests. Two main recovery mechanisms were focused on as metabolites from these microbes could prevent viscous fingering as a result of biopolymer production from G2 and interfacial tension reduction through the production of biosurfactants from G1. The action of these microbes on crude oil samples from the Niger Delta region was also investigated. Results from the analysis showed a considerable decrease in certain fluid properties such as gel strength and viscosity. However, this study has shown conclusively that considerations must be given to the type of oil reservoir and its characteristics before choosing a potential microbial candidate for the recovery process.

Index Terms— Bacillus polymyxa, Biopolymers, Biosurfactants, MEOR, Pseudomonas earuginosa, Gel strength, Viscous fingering.

1 INTRODUCTION

The world energy demand is ever increasing, although this current energy demand is satisfied petroleum fuel, a significant fraction is still left unexplored after primary reservoir energy must have depleted. Most mature oil reservoirs still have approximately 40 – 60% of the original oil in place unproduced as a result of production inefficiencies [1], [2]. In 2001, the U.S. Information Administration reported that between 1994 and 2003, over 17.5million reservoir barrels of oil was lost as a result of premature abandonment of marginal oil wells. The water flooding process of oil recovery has proved a worthy alternative but is faced with a number of reservoir challenges such as viscous fingering and inability to effectively tackle the problem of oil- rock interfacial tension. On this basis, a number of other tertiary recovery mechanisms have been explored which includes surfactant flooding, polymer flooding, thermal application, gas injection etc. and most recently, microbial EOR [2], [3].

Microbial enhanced oil recovery is a multi-disciplinary field incorporating among others geology, chemistry, microbiology, fluid dynamics, petroleum engineering and chemical engineering. It is a biological based technology that entails the manipulation of function or structure or both of microbial environments existing in oil reservoirs for the purpose of improving the recovery of oil entrapped in the porous media with tolerable economic implications [1].

It is imperative to apply various methods and processes for artificially increasing production of crude oil from semi-depleted reservoirs. The idea of using bacteria for the production of oil from depleted oil wells was first suggested by Beckman back in 1926, [1], [2], [3], [4]. When these bacteria are injected, they also will initially flow preferentially into high-permeability zones with the injected water, but then will either grow or produce biopolymers to plug those zones, preventing viscous fingering [2], [4], [5] and also in some cases reduce the interfacial tension (IFT) between oil and water interface to enhance fluid mobility [3], [5], [6], [7].. Another factor affecting bacterial penetration is the negative charge on the cell surface. This charge, however may affect both mobility and penetration of the bacteria [8]. Prominent among problems in oil recovery is the occurrence of inefficient sweep actions in the reservoir as a result of viscous fingering. Also, a retarded mobility property of residual oil as a result of persistent interfacial tension in the reservoir may result in the recovery of insignificant volume of oil. This study tends to investigate the applicability of certain bacteria` to tackle these problems. However, a series of biochemical investigateons must be made so as to ascertain the adaptation and performance of these microorganisms in reservoir conditions [9], [10].

2 RESEARCH METHODOLOGY

Contaminated water samples served as the inoculums for this investigation which was brackish water collected in a sterile container from a Niger Delta location. This sample was collected and transported to the Biology Laboratory in Rivers State University of Science and Technology, Port Harcourt, Nigeria.

2.1 Sterilization of glass wares and media used

All glass wares were washed and cleaned with clean water. Perti-dishes, conical flasks, test tubes were all sterilized in an oven at 160^{o}C for 1-3hours. The media used and distilled water for serial dilution were autoclaved at 15psi at a temperature of 121^{o}C for about 15 minutes. Media was prepared by weighing a 28g of nutrient agar into 100ml Erlenmeyer flask. This was dissolved in 1litre of distilled water and broth to boiling in order to dissolve completely. Sterilized at 121^{o}C for 15minutes at a pressure of 15psi, the media was allowed to cool to about 45^{o}C and about 15ml of the medium was poured into sterile petri dishes. These plates were allowed to set and the surface of the medium dried in an oven for about

15minutes before use.

2.2 Preparation of Mineral salt Agar Medium

Mineral salt Agar Medium used was prepared using the following chemical composition;

K_2HPO_4	0.5g
$MgSO_4HPO_4$	0.3g
K_2HPO_4	1.0g
NaCl	0.2g
$MnSO_4..7H_2O$	0.02g
FeSO4.6H4O	0.03g
N_aNO_3	0.03g
ZnCl	15.0g
Agar agar	0.5g
Glucose	1.5g
Sucrose	1.0g
Maltose	1.0g
Peptone	3.5g
Starch	5.0g
Distilled water	200ml

2.3 Diluent Preparation

Diluent was prepared by dissolving 0.85g of NaCl in 100ml of distilled water. 9ml of the normal saline (diluents) was pipetted and transferred into different test tubes and were sterilized, autoclaved at 121°C for about 15 minutes at a pressure of 15psi. Allowed to cool, it was then used in the serial dilution of the contaminated water sample.

2.4 Serial dilution of samples and Identification of bacteria isolates

1ml of homogeneous mixed sample was transferred using sterile 1ml pipette into sterile test tubes containing normal saline as diluents. 1ml of the sample was transferred to other 9ml diluents and mixed properly. This ten-fold serial dilution continued until the required dilution was obtained. For isolate identification, isolates were obtained and subjected to various characterization procedures. Pure bacteria isolates were identified on the basis of their culture, morphological and physiological characteristics. Certain tests were performed to properly identify these microorganisms.

2.5 Microbial Identification Tests

i. Graham Staining reaction

This Test was carried out with the procedures stated in [10]. Smears of isolates were made on microscopic glass slides, air-dried and heat fixed by passing the slides over a bunsen flame. The fixed smear was stained with crystal violet for 30 seconds, the excess water was poured out and was gently washed off with running water. Finally, the smear was flooded with iodine for 20-30 seconds. The smears was again washed off with running water and with 95% ethanol until the color of the crystal violet no longer dipped. Finally, they were counter stained with Safranin for 10 seconds and washed off with water. The smear was bottled, dried and observed under the oil immersion object of the light microscope. The positive organisms picked up the purple-blue color of the crystal violet, whereas the negative organisms picked up the pink color of the safranin.

ii. Motility Test

This test was conducted to determine the presence and absence of flagella as organelles of motion in the bacterial isolates as described by Cruikshank in 1975, [10]. 10gram of peptone and 4g of agar were weighed and put into a conical flask and 1litre of distilled water added to it. The mixture was heated to ensure proper dissolution, and then 10ml of the mixture was dispensed into different test tubes plugged with cotton and autoclaved at 121°C. The test tubes were allowed to cool and set in an upright position/vertical position. These tubes were inoculated by stabbing, incubated at 37°C for 48hours. Motility test was selected by diffusing growth that spread throughout the medium while non-motile grew only along the stab line.

iii. Oxidase test.

The wet filter paper method used in [10] was adopted and was conducted to demonstrate the presence of oxidase enzymes in the isolates which will catalyze the transport of electrons between electron donors in the bacteria and a redox dye tetramethyl-p-phenylene-diamine. A small portion of the isolates (24hrs culture) was smeared on the part of the filter paper strip impregnated with freshly prepared oxidase reagent. The reaction was observed for 10 seconds to note every color change. A positive reaction was indicated by an intense deep purple coloration appearing within 5-10seconds, a delayed positive reaction was indicated by no color change.

iv. Catalase Test:

The slide method as described [10] was adopted for the demonstration of the presence of catalase, an enzyme that releases oxygen from hydrogen peroxide. Discrete colonies of over-night cultures of isolates were placed in microscopic glass slides and few drops of hydrogen peroxide were added to the cultures of the glass slides. A positive reaction was indicated by the production of gas bubbles while no bubble indicated a negative result.

v. Coagulase Test

This test was conducted to detect bacteria capable of coagulation when reacted with human or animal plasma. A drop of water was placed on two clean microscopic slides and a colony of a test organism was emulsified on each of the drops to make two suspensions. Then a drop for plasma was added to a portion of the suspension and then mixed gently to observe the clumping within 10 seconds while the other served as control.

vi. Starch Hydrolysis

This test was to determine the ability of the isolates to hydrlyse starch.5g of soluble starch and 7g of nutrient agar were weighed into a cornical flask and 250ml of distilled water added, sterilized and autoclave at 121°C for 15minutes and finally poured into sterile petri dishes. The organisms were streaked on the plates and incubated at 37 °C for 48hours after which

plate were flooded with Lugol's iodine solution. The medium turning blue where starch has been hydrolyzed was an indication of the presence of starch.

vii. Urease Test

This test was conducted to determine the decomposition ability of bacteria on urea. 1g of peptone, 5g of NaCl, 2g of K_2HPO_4 20g of agar and 6ml of phenol red(1 in 500 aqueous solution) were weighed and added into 1litre of distilled water. Also 10ml of 10% glucose solution and 100ml of 20% urea solution were sterilized separately by filtration. The basal medium was prepared without the glucose and urea which was sterilized by autoclaving in a flask at 121°C for 15 minutes. This was cooled to about 50°C. The glucose and solution were then added in the medium tubes. Test isolates of overnight cultures inoculated heavily over the entire sloped surface at 37°C for 96 hours. Change in color to intense pink indicated positive results while negative results will maintain initial coloration.

viii. Indole Test

This was done to demonstrate the ability of certain bacteria to decompose amino acid trytophane to indole which accumulates in the medium. 10g of peptone and 2.5g of NaCl were weighed and poured into conical flasks containing 500ml of water. The mixture was dispensed into test tubes and autoclaved at 121°C for 15 minutes test isolates were inoculated into the medium and incubated for 48Hrs at a temperature of 37°C. 0.5ml of kovac's reagent was added and shaken gently. A red color in the alcohol layer indicated a positive reaction while negative result was indicated by a brown color at the upper layer of the medium.

ix. Citrate utilization test.

This is a test for the ability of an organism to utilize citrate as the sole carbon and energy source for growth and ammonium salt as the sole source of Nitrogen using the Koser's medium. The medium was dispensed into test tubes and sterilized by autoclaving at 121°C. The medium was allowed to cool and the test isolates were inoculated and incubated at 37°C for 96 hours. A positive reaction was indicated by a dense turbidity ie growth.

x. Nitrate reduction Test

This was to test for enzyme nitrate reduction in the presence of suitable electron donor, to a nitrate which can be tested by an appropriate colorimetric reagent. A 0.2g potassium nitrate and 5g of peptone were added to 1litre of distilled water. 5ml of the mixture was dispensed into test tubes and autoclaved at 121°C for 15 minutes. The test tubes were allowed to cool and inoculated with overnight cultures and incubated at 37°C for 96 hours. 0.1ml of the rest of the reagent was added to the test tubes. A red color developed within a few minutes indicated the presence of nitrate. Zinc powder was also added to clearly indicate the reduction of nitrate to nitrogen gas.

xi. Methyl red Test

This is the ability of organisms to produce and maintain stable acid end products from glucose fermentation. Buffering glucose broth was inoculated with overnight cultures and incubated at 37°C for 48 hours. A few drops of methyl red reagent were added to the solution shaken and read immediately; positive reaction was indicated by a bright read coloration while a negative reaction was indicated by a yellow coloration.

xii. Voges-Proskauer Test

This test is for the detection of the production of acetylmethyl-carbinol (acetoin), a natural product from pyruvic acid in the cause of glucose fermentation. Buffered solution broth was inoculated with the test organism and incubated at 37°C for 48 hours. 3m of alpha-naphthol and 1ml of 40% KOH were added and shaken thoroughly. A positive result was indicated by the development of a pink coloration and no color change was conclusive on a negative result.

xiii. Carbohydrate fermentation test

This test is conducted to determine the ability of bacteria to metabolize carbohydrate. The sugars used were glucose, sucrose, lactose, maltose, mannitol and arabinose. 15g of peptone was dissolved in 1litre of distilled water. Also 25ml of 0.2% Bromocresol purple was added and then 10g of sugar was also added and shaken thoroughly. 5ml of this solution was dispensed into test tubes containing inverted Durham tubes and plugged with cotton wool, the tubes containing the medium were autoclaved at 121°C for 10 minutes. The sterilization process ensured the removal of bubbles from the Durham tubes. The test tubes were inoculated with a loop full of the overnight cultures and incubated at 37°C for 24.48 hours and observed critically. Results indicated acid production which changed the color of the indicator from purple to yellow and gas production (displacement of liquid volume in the Durham tube). A negative result will not show any of these results. Results of these tests will be presented and the effects of these isolates will also be investigated on crude oil samples.

3 Result and Discussion

3.1 Identification of Isolates

After series of microscopic examination, four isolates were identified to be potential biopolymer and biosurfactant producers which are *Pseudomonas aeruginosa, Bacillus polymyxa, Proteus sp* and *Micrococous sp* with a nomenclature G1, G2, G3 and G4 assigned respectively.

TABLE 1
ISOLATE IDENTIFICATION RESULTS

ISOLATES	G1	G2	G3	G4
Cell morphology	rod	rod	rod	cocci
Gram reaction	-ve	+ve	-ve	+ve
Motility test	+ve	-ve	+ve	-ve
Spore formation	-ve	-ve	+ve	-ve
H_2S production	-ve	+ve	-ve	+ve
Methyl red	-ve	-ve	+ve	-ve
Voges-proskauer	-ve	+ve	+ve	+ve
Oxidase	-ve	-ve	-ve	+ve
Indole	-ve	-ve	+ve	-ve
Citrate utilization	+ve	-ve	-ve	-ve

Urease	-ve	-ve	+ve	-ve
Catylase	+ve	+ve	+ve	+ve
Starch hydrolysis	-ve	+ve	-ve	-ve
Nitrate reduction	+ve	-ve	+ve	-ve
Glucose	-ve	-ve	-	-
Lactose	-ve	-ve	+ve	-
Maltose	-ve	-ve		-
Sucrose	-ve	-ve	-	-
Identification	*Pseudo-monas aeruginosa*	*Bacillus polymyxa*	*Proteus sp*	*Micrococous sp*

3.2 Characterization of Isolates

Characterization and identification of isolates was based on their culture morphology, microscopic examination, carbohydrate fermentation and other biological tests. Consequently (4) bacteria were identified at the end of the process under aerobic conditions. However, emphasis will be placed on *Bacillus polymyxa* and *Pseudomonas aeruginosa.*

A. Bacillus polymyxa (G2)

i. Solubility, Viscosity and Alkalinity

Bacillus polymyxa (G2) was soluble in a medium of pH greater than 11.4 and insoluble in nano-pure water but insoluble when heated to 50oC. When the pH of the biopolymer solution was reduced to 10.8, a viscous free-flowing gel was formed. Also, when an insoluble biopolymer mixture was made soluble on application of heat, a semi solid gel formed on cooling. G2 was also viscous in nature with a viscosity of 1.17cp at 6rpm. This was confirmed with a Brookfield viscometer model (K34751), DV1+ viscometer with spindle 4, from the Petroleum Engineering laboratory in Rivers State University of Science and Technology. The sample volume tested was about 80ml. It was observed that its alkalinity varied with changes in pH. This was confirmed using the pH meter. The effect of pH on viscosity of the microbe was made obvious when the viscosity increased at a drop in pH below 10.8. A further reduction in pH below 6.0 resulted in a decrease in biopolymer solution.

ii. Temperature

Properties of Bacillus polymyxa (G2) changed with variation in temperature. The reduction in viscosity as a function of temperature is presented in the figure below. It also displays the relationship of viscosity and temperature of a 10% biopolymer solution with an initial viscosity of less than 20cp and initial pH of 11.4. The temperatures of investigation were at 25°C, 50°C, 75°C and 95°C.

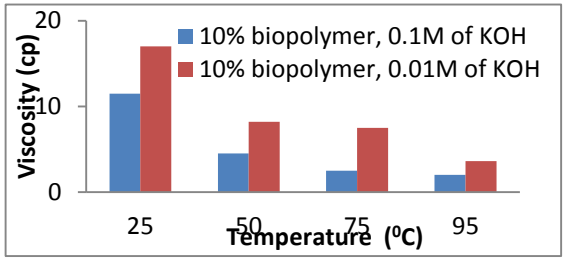

Fig. 1. Biopolymer viscosity as a function of Temperature

i. Viscosity, Alkalinity and Temperature

Pseudomonas aeruginosa (G2) was observed to have a viscosity of 1.25cp at 6rpm, however, this was confirmed with a Brookfield viscometer. The pH of the isolates without adjustment was 7.0. Varying pH levels were found to alter surface tension property of the isolates at values less than 6.0 as shown in the figure 2 Temperature variations from incubating isolates at a range of 21 - 70⁰C was investigated and these variations were found to have a considerable effect on surface tension properties as well.

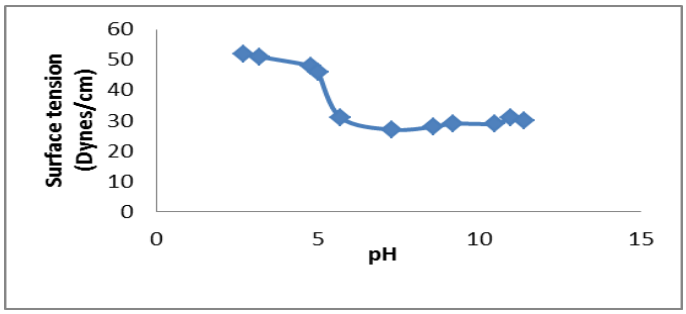

Fig. 2. Surface tension as a function of pH for biosurfactant

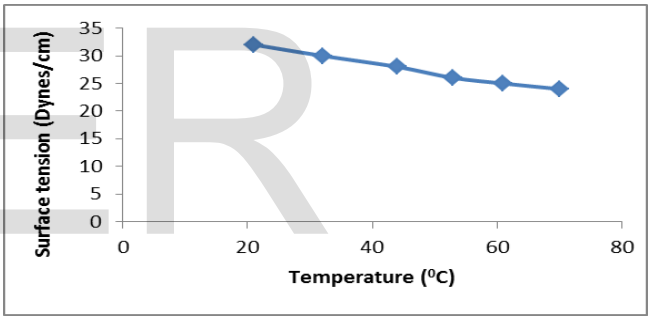

Fig. 3. Reduction inSurface tension with increase in temperature

3.3. Applicability of Investigated Isolates on Crude Oil Samples

Crude oil sample collection was in collaboration with field technicians from the wellheads of some producing locations in the Niger Delta region. Three samples were collected from three different oil producing locations. These samples were properly labeled for easy identification. Collection bottles were properly cleaned and rinsed with the samples to be contained. The crude oil samples were then assigned the nomenclature X, Y and Z for easy identification. The physiochemical properties of the crude oil samples are presented below.

TABLE 2

PHYSIOCHEMICAL PROPERTIES OF CRUDE OIL SAMPLES

Crude oil samples	T (^{0}C)	Viscosity at 40^{0}C (cp)	API \Gravity	Specific Gravity	% Sulphur Content	% H_2O content	% Salt Content	Gel Strength (lb/ft^2)
X	25.5	4.92	42.0	0.46	0.13	0.01	0.45	11.78
Y	24.3	5.23	43.5	0.80	0.12	0.01	0.44	9.78
Z	28.4	1.46	44.4	0.80	0.13	0.01	0.39	5.6

TABLE 3
CONCENTRATION OF HEAVY METALS ON CRUDE SAMPLES

Crude oil samples	Zn	Cu	Pb	Fe	Mn	Co	Cd	Ni	V
X	0.01	0.04	0.05	0.22	0.30	-	0.01	3.20	0.77
Y	0.01	-	-	0.27	0.41	-	-	6.45	7.60
Z	0.01	-	-	0.18	0.35	-	-	2.01	0.50

3.4. Applicability of Investigated Isolates on Crude Oil Samples

A. Action of G1 on crude oil samples

Two sets of beakers were filled with 50ml of X, Y and Z samples respectively. The first sets of samples were injected with 20ml of *Pseudomonas aeruginosa* alone while the second sets of beakers each with the samples X, Y and Z respectively were injected with 20ml of microbial solution with additional nutrient broth. It was observed that there was a reduction in viscosity of the various samples of crude oil, but that which contained nutrient broth recorded a decrease in viscosity as a result of rapid metabolite production.

B. Action of G2 on crude oil samples

The same procedure was followed for the G1 isolates, but for this case, *Bacillus polymyxa* was used and the gel strength of crude oil samples increased for the first set of beakers, a more intense and rapid gel strength increment was recorded in the second set of beakers containing the nutrient broth. This was as a result of the expected microbial metabolite production.

Consequently, the performance of the microbial activity is a function of the quality of the nutrient broth. After a 24hours investigation, it was observed that there were no remarkable changes in the beakers containing of microbes and oil sample alone for samples X, Y and Z. The changes as noticed in the beakers containing microbes, nutrient broth and crude oil samples were considerable and results from this investigation is presented in the tables below

TABLE 4
ACTION OF G1 ON CRUDE OIL SAMPLES

Crude oil samples	microbe	volume without nutrient broth (ml)	Volume withnutrient broth (ml)	Incubation time (hrs)	Initial Viscosity (cp)	Final Fluid Viscosity (cp)	
						Without nutrient broth	Without nutrient broth
X	G1	20	20	24	4.92	4.80	4.56
Y	G1	20	20	24	5.23	5.19	5.02
Z	G1	20	20	24	1.46	1.38	1.09

TABLE 5
ACTION OF G2 ON CRUDE OIL SAMPLES

Crude oil samples	Microbe	Volume without nutrient broth (ml)	Volume with nutrient broth (ml)	Incubation time (hrs)	Initial gel strength (lb/ft^2)	Final gel strength (lb/ft^2)	
						Without nutrient broth	Without nutrient broth
X	G2	20	20	24	11.78	12.12	12.84
Y	G2	20	20	24	9.78	10.14	10.86
Z	G2	20	20	24	5.60	6.01	6.89

These investigations have explored the description of biopolymers and biosurfactants through isolation and series of biochemical tests. At the end of the entire process, four isolates

were identified; *Pseudomonas aeruginosa, Bacillu*s polymyxa, *Proteus sp* and *Micrococous sp* assigned G1, G2, G3 and G4 respectively. Based on their biocompatibility, biodegradability and amphipathic nature, G1 and G2 were selected as potential candidates for biosurfactant and biopolymer production respectively. Their viscosities, pH and temperature tolerance values confirmed their capacity to act as mobility control and emulsifying agents. **G2** prevents viscous fingering by increasing the viscosity of the displacing fluid and also, preventing hydrodynamic instability that may set in if a less viscous fluid tries to displace a more viscous fluid, hence improving sweep efficiency. **G1** on the other hand have been confirmed to possess the ability of comfortably lowering Interfacial tension of the Mineral Salt medium from 63dynes/cm to 29dynes/cm after a 48hours incubation period. The action of **G1** on gel strength and **G2** on viscosity on three different crude oil samples from the Niger Delta region showed that microorganisms isolated from local environments have the capacity to effectively recover residual oil in a cost effective manner from these reservoirs. Being biodegradable and environmentally friendly, biosurfactants also abates the presence of heavy metals from the surroundings as demonstrated by Patil *i*n 2012 [11].

4 CONCLUSION AND RECOMMENDATION

This study have shown conclusively that many reservoir characteristics must be determined first before applying MEOR, some of which are porosity, permeability, salinity, reservoir temperature and pressure. It is uncertain that a single MEOR method can be applied to all types of reservoirs, a reservoir that can allow injected bacterial to spread faster and more efficiently to generate metabolites will be appropriate for this process. However, engineers must adhere to the following considerations for an effective MEOR process;

- MEOR method should be specified for specific reservoirs.
- Considerations should be made to the effects of reservoir conditions before the MEOR scheme.
- Data on the performance of biosurfactants and biopolymers compared with that of synthetic surfactants and polymers under reservoir conditions should be obtained.
- Potential environmental effects of introducing microbes into reservoirs should not be ignored in the quest for enhancing oil recovery
- Since crude oil samples from the Niger Delta could be a source of heavy metals particularly Ni and V, and independent radiation and safety consultant must take responsibility of evaluating the tolerant level of these heavy metals in the producing region.
- Nutrient broth is a more suitable nutrient medium to grow and maintain the production of expected metabolites and microbial population and hence must be injected with selected microbes.

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