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Influence of Growth Media on Hydrphobicity of Phenol-Utilizing Bacteria Found in Petroleum Refinery Effluent

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Abstract

The effect of growth medium on hydrophobicity of phenol-utilizing bacteria isolated from petroleum refinery effluent was investigated. The hydrophobicity expression of the isolates were assessed via BATH, SAT and CRB assays. Four different growth media: tryptone soy broth (TSB), nutrient broth (NB), peptone water (PW) and Bushnell Haas broth (BH) was used. All the test isolates exhibited high to moderate hydrophobicity when grown on all the media. However, using SAT assay, Pseudomonas sp. RWW was found to be strongly hydrophobic (<1.0) when grown in all the media while Escherichia sp. OPWW was found to be moderately hydrophobic (1.0 – 2.0) in all the media using the same SAT assay. Using Congo red binding assay, Bacillus sp. RBD was found to express weak hydrophobicity when grown in BH medium with Congo red up take value of 9.0 µg. All the media used for the assays were observed to be quite well for expression of hydrophobicity by refinery effluent bacteria in the following order: TSB > HB > PW > BH.

Keywords: Phenol-utilizing bacteria, Refinery effluent, Hydrophobicity, Growth media.

Introduction

Oil refinery is one of the major industries in the petroleum industrial world. It is where other industries, especially, petrochemical industries rely for their feedstock in which the resulting processed products such as synthetic materials and resins are useful for agricultural purposes[1]. Processing of raw crude oil in the refineries means that the oil will pass through many processes for the production of the desired finished product. The processes of producing these refined products have resulted in the production of volumes of wastewaters[1]. Common sources of these wastewaters include water from storage tanks and processing equipments. The composition of these wastewaters varies according to the type of raw material processed and the treatments employed in reducing wastewater contaminants[2,3]. Wastewater from oil refinery industries usually harbours toxic chemicals such as hydrocarbons, phenols and heavy metals among others. Due to its chemical composition and concentration, their effects on the environment are not desirable (eutrophication) as well as being dangerous to microbial and human health.

The toxic effects of refinery effluents on bacteria strains have been reported[4,5]. Bacteria are unable to insulate themselves from the toxic nature of their habitat because of their large surface area that are exposed to these harsh environment as concerned with industrial effluents especially refinery effluents. The pollutants of the effluent exert their toxic effects on the bacterial surfaces thereby inhibit their attachment to substrates and other surfaces. The most important mechanism of this action is the destabilization of cell membrane[6]. This results in adaptation and changes in their physiological functions.

The interfacial role of microbial cells is associated with its physicochemical properties and chemical composition. Cell contact with the environments is by cell wall which plays important role in microbial life. Most organisms are in contact with water and are always in aqueous phase as a result of hydrophilic moieties that surrounded microbial surfaces[7]. The hydrophilic sites of bacteria cellwall consist of charged groups such as carboxyl, phosphate, amino and guanidyl groups and the non charged hydroxyl group while the hydrophobic consists of lipids and lipopolysaccharides[7,8].

Hydrophobic responses are known to be responsible in the adherence of microorganisms to several of surfaces in the environments. The partitioning activities of bacterial cells at interfaces is related to the hydrophobic nature of microorganisms hence the adherence of bacteria to surfaces, marine sediment, to one another as well as growth on hydrophobic compounds[9-11]. Cell surface hydrophobicity relating to bacterial adhesiveness has been documented[11]. Doyle[12] and Rosenberg[13] reported that microbial cell surface hydrophobicity varies from organism to organism, from strain to strain and is influenced by the growth medium, bacterial age, growth temperature, pH, ionic strength and cell numbers. As a result of importance of microbial hydrophobicity in fermentation technology[14], engineered bio-treatments of water[15], etc, several techniques of measuring microbial hydrophobicity had been documented[16]. These include bacterial adhesion to hydrocarbons (BATH), salt aggregation test (SAT), Congo-red binding to bacteria (CRB), micro-sphere adhesion to cells (MAC), etc[17-19].

In this study, we assess the influence of growth media on the hydrophobicity of bacterial species isolated from Port Harcourt oil refinery effluents treatment plants.

Material and Methods

Sample source and organisms: Samples and its collections were described elsewhere[20]. Samples include physicochemically treated raw wastewater (RWW), biologically treated wastewater (Rotary biodisk) (RBD), observation pond treated wastewater (OPWW) and discharge pipe wastewater (DP). The physicochemical analyses are as shown in table 1. The organisms were isolated from the samples using mineral salt-phenol agar medium[4] and were marked according to their sources as well as being stored in agar slants at 4°C.

Preparation of inoculum and culture condition: The organisms were grown in the following sterile media: Tryptone soy broth (TSB) (Oxoid, Basingstoke, UK), Nutrient broth (NB)(Fluka), Peptone water (PW) (Sigma Aldrich, Germany) and Bushnell Haas mineral salt broth (BH) medium amended with phenol (50 mg/l) contained in 250ml Erlenmeyer flasks covered with cotton wool wrapped in Aluminum foil. This was done by inoculating the flasks with organisms scrapped from the agar slants with sterile wire loop. The flasks were incubated on a rotary shaker (120 rpm) for 24h at 28 ± 2 °C. The cells were recovered by centrifugation (6,000 rpm for 10 min) and washed twice in phosphate buffered saline (PBS, 0.02M; pH 7.2 for BATH assay and Congo red assay and pH 6.8 for SAT), thereafter resuspended in the same medium. The turbidity was brought to 1.0 by spectrophotometrical adjustment at 540nm.

Bacterial hydrophobicity: Surface hydrophobicity of phenol-utilizing bacteria was assessed using the bacterial adherence to hydrocarbon (BATH), modified salt aggregation test (SAT) and Congo red binding.

Bacterial adherence to hydrocarbon: BATH was performed as described elsewhere[13] with little modification. The cell suspensions (A_0) were dispensed in 4ml aliquots into sterile 20ml volume screw capped test tubes. The tubes received different volumes viz 0.1, 0.2, 0.3, 0.4 and 0.5ml of either *n*-octane or p-xylene (Sigma Chemical Co., St. Louis, Mo., USA). The mixtures were vortexed uniformly for 120s and allowed to equilibrate for 15 min for the completion of biphasic formation. Thereafter, the aqueous phase was carefully recovered and the optical density estimated at 540 nm, (A_1). Values were expressed as the percentage of bacteria adhering to the hydrocarbons (A) relative to the control (A_O) as follows:

$$A\ (\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

The reference value for the BATH assay is the percentage of bacteria from 4 ml of suspension that partition into 0.5 ml of *n*-octane or *p*-xylene. Strongly hydrophobic, moderately hydrophobic and hydrophilic were assigned to the organisms when percentage of adhesion values were > 60%, 40-60% and <40% respectively[21].

Salt aggregation test: SAT assay was carried out as described elsewhere[22] with little modifications. The assay is based on clumping of bacterial in the presence of salts[16]. Isolates were salted out (aggregated) by combining 25µl volumes of the cell suspension with equal (25µl) volumes of a series of varying molarities (M) of $(NH_4)_2SO_4$ solution (0.2 - 4.0 M, differing by 0.2) in different wells in a microplate. Addition of 400 µl of 0.1% w/v methylene blue solution to 10 ml volumes of $(NH_4)_2SO_4$ solution facilitates better visualization of aggregation[21]. The plate was rocked for 4 min after which it was visually examined and scored against a white background for cell aggregation. The reaction mixture causing maximum agglutination was considered positive whereas absence of agglutination was considered as negative. Lowest concentration of $(NH_4)_2SO_4$ in the reaction mixture causing clumping of cells is expressed as hydrophobicity. Classification was expressed as: < 1.0 M = strongly hydrophobic, 1.0 – 2.0 M = Hydrophobic, > 2.0 M = Hydrophilic[20].

Congo red binding assay: This assay is used to study the pigment binding ability of the strains as well as a marker of hydrophobicity[23]. The experiment was performed as described elsewhere[24] with little modification. Aliquot (1.0 ml) of bacterial suspension were dispensed into screw capped glass test tubes containing 4 ml of PBS amended with 25µg/ml of Congo red dye and incubated at 28±2°C for 15 min. Thereafter, the Congo red bound to cells are removed by centrifugation at 6,000 rpm for 10min. The supernatant (cell free Congo red solution) was collected in separate tubes and its absorbance determined spectrophotometrically at 480nm against a PBS blank. The amount of Congo red dye that bind to the cells were calculated from a standard curve as the difference between the amount added to the mixture and the amount remaining in the cell free Congo red solution. Uptake of Congo red greater than 10 µg was scored as strongly hydrophobic[19].

Results and Discussion

The physicochemical properties of the petroleum refinery effluent samples are shown in table 1. The refinery effluent samples had increased concentrations of THC, BOD, COD and phenol as all measured values exceeded the Federal environmental protection (FEPA) permissible limit. This showed that autochthonous microorganisms isolated from these samples are subjected to pollutants stress. The organisms isolated from the samples include *Pseudomonas* sp. RWW, *Bacillus* sp. RBD, *Escherichia* sp. OPWW and *Corynebacterium* sp. DP. These strains represent the major morphotypes present in their respective sources. These organisms are well known for their ability to grow on highly hydrophobic compounds of petroleum origins[25].

Figure 1 showed a gradual decrease in absorbance of the cell suspensions after mixing with varying volumes of hydrocarbons (*n*-Octane and *p*-Xylene). This indicated that the cells were partitioned into the hydrocarbon phase and the loss of bacteria from the aqueous phase was approximately proportional to the volume of *n*-octane and *p*-xylene added to the cell suspension. Similar report had been observed by Sorongon[26] in their work using *Cytophaga* sp. strain U67 to assess hydrophobicity of gliding bacteria. Also, Lachica and Zink[27] obtained similar results in their work when they assess cell surface charge and hydrophobicity of *Yersinia enterocolitica*. The organisms partitioned more in *n*-octane than in *p-xylene* as shown in Figure 1. This may be as a result of devastating effect of *p-xylene* on the surface of the organisms than *n*-octane regardless of growth medium of the organisms. In a similar study, Pembrey[28] as well as Lachica and Zink[27] attributed the low partitioning of the organisms in *p*-xylene to lysing effect of the compound on the organisms.

Results of cell surface hydrophobicity of the organisms using BATH, SAT and Congo red binding assays are shows in table 2. Using *n-octane* in BATH assay, *Corynebacterium* sp. DP was found to be moderately hydrophobic when grown in PW and BH media respectively while other organisms expressed strong hydrophobicity when grown in all the media. This indicated that hydrophobicity could not be better expressed when *Corynebacterium* sp. DP were allowed to grow in PW and BH but will be better expressed in NB and TSB respectively. All the test organisms were observed to express high hydrophobicity when grown in TSB then followed by when grown in NB medium (table 2). This may be attributed to a high production of hydrophobic cell surface proteins[29,30]. However, the finding in this study is in line with the observation of Das and Kapoor[31] who obtained high hydrophobicity with *Staphylococcus aureus* by BATH when the organism was grown in TSB and PW.

Using *p*-xylene in BATH assay, all the organisms expressed moderately hydrophobicity when grown in TSB, NB, PW and BH media respectively. Only *Pseudomonas* sp. RWW was observed to express strong hydrophobicity when grown in TSB medium. The moderately hydrophobicity expression by the organisms in BATH using *p*-xylene may be as a result of alteration on the surface of the organisms by toxic effect of *p*-xylene.

Cell surface hydrophobocity estimated by salt aggregation test (SAT) assay as shown in table 2 showed that majority of the test isolates expressed moderate hydrophobicity when grown in all the media. This indicated that the bacterial strains found in Port Harcourt refinery effluent are moderately hydrophobic. The differences in SAT values between *Pseudomonas sp*. RWW that showed strong hydrophobicity in all the growth media than other test organisms may be as a result of differences on cell surface charges. This indicates that SAT values may be dependent on the charge on microbial surface as well as the age of the culture no matter the type of growth media. This is in agreement with the results obtained by Qadri[21] in which they found that aggregation of bacterial strains increase with old cultures as charges on microbial surfaces increases.

Using Congo red binding assay, the results obtained showed that the test organisms bind effectively with Congo red. This indicated that the organisms are hydrophobic and can uptake Congo red in solution effectively irrespective of growth medium. It was only *Bacillus* sp. RBD that showed weak Congo red uptake with a value of 9.0 µg when grown in BH medium indicating weak hydrophobicity. *Pseudomonas* sp. RWW showed the highest Congo red uptake with a value of 14.9 µg when grown in NB medium. The hydrophobic nature of the organisms may be as a result of their growth in non nitrogen limitation media as nutrient starvation affects cell surface hydrophobicity when bacteria are cultivated under nitrogen limitation medium[32].

Table -1
Characteristics of the petroleum refinery wastewater

Parameter/unit	Sample source			
	RWW	**RBD**	**OPWW**	**DP**
pH	7.64	8.18	7.45	8.87
Temperature ^{o}c	26.4	26.1	26.8	26.7
Elect. Conduc.(µscm^{-1})	845	443	926	643
Oil and grease (mg/l)	17.5	15.0	21.0	16.0
BOD (mg/l)	32.0	8.0	12.8	12.8
COD (mg/l)	112.0	76.0	114.0	84.0
PO_4 (mg/l)	0.22	0.14	0.13	0.12
SO_4 (mg/l)	37.63	13.52	35.3	11.8
Phenol (mg/l)	71.2	13.6	10.1	9,4
Pb (mg/l)	<0.01	<0.01	<0.01	<0.01
Zn (mg/l)	0.13	0.02	0.06	0.08
Cu (mg/l)	<0.01	<0.01	0.01	0.01

Legend: RWW: Raw wastewater, RBD: Rotary biodisk, OPWW: Observation pond wastewater, DP: Discharge pipe.

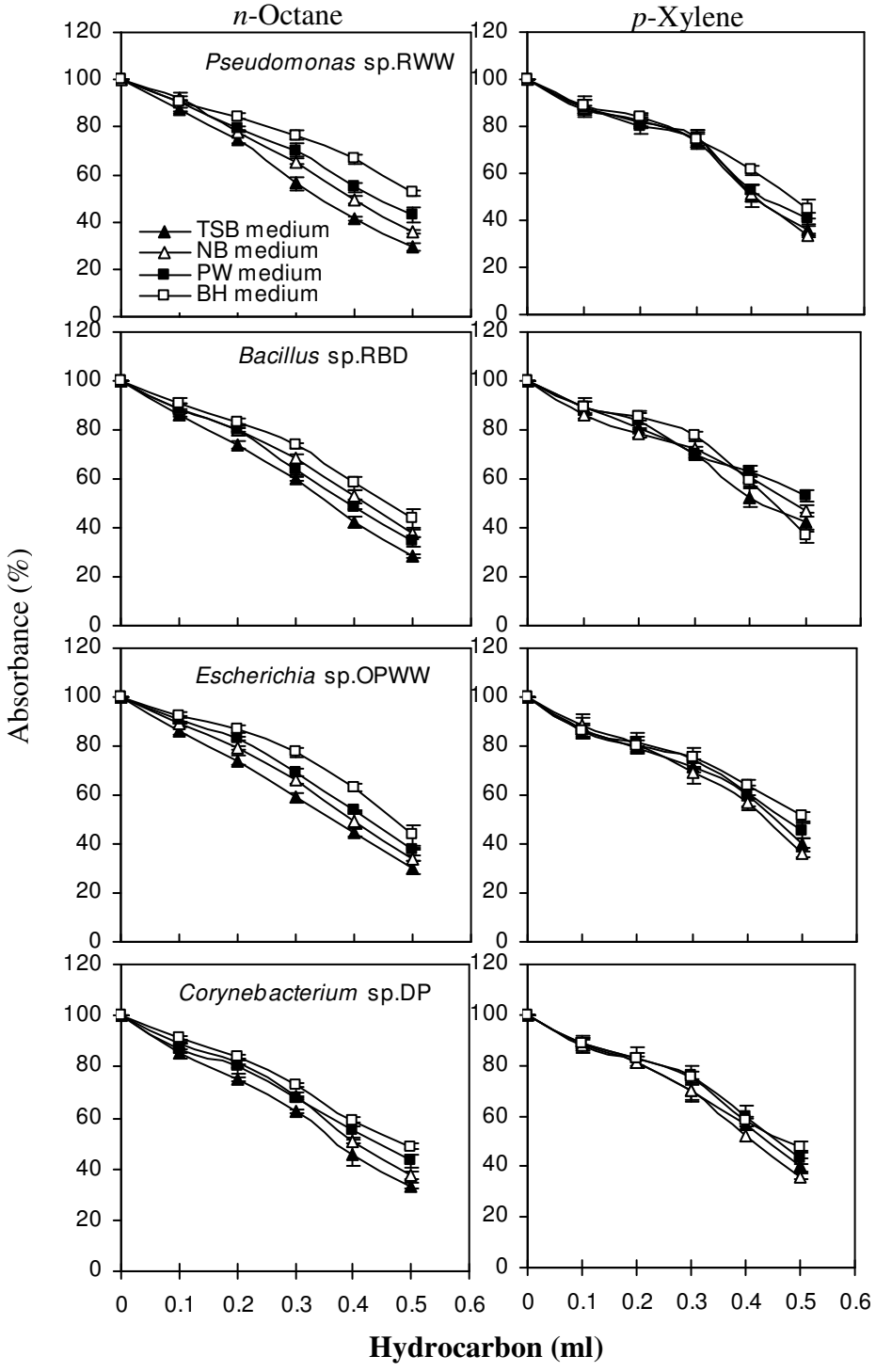

Figure-1
Percentage absorbance of aqueous suspension of bacteria strains remaining after being mixed with increasing volumes of hydrocarbon of (A) *n*-Octane and (B) *p*-Xylene

Table- 2
Cell surface hydrophobicity of phenol-utilizing bacteria grown in different growth media

	Hydrophobicity			
	BATH (%)		SAT	Congo red binding
Bacteria/medium	***n*-octane**	***p*-xylene**	**$M(NH_4)_2SO_4$**	**(µg)**
***Pseudomonas* sp. RWW**				
TSB	66.2 ± 1.0	61.3 ± 1.0	0.4 ± 0.0	13.3 ± 1.0
NB	62.3 ± 1.0	55.8 ± 4.0	0.6 ± 0.0	14.9 ± 1.0
PW	62.0 ± 1.0	58.2 ± 3.0	0.6 ± 0.0	11.1 ± 1.0
BH	60.3 ± 2.0	56.7 ± 5.0	0.4 ± 0.0	13.3 ± 0.0
***Bacillus* sp. RBD**				
TSB	65.6 ± 1.0	58.3 ± 3.0	0.2 ± 0.0	12.1 ± 0.0
NB	64.0 ± 1.0	53.0 ± 2.0	1.8 ± 0.0	10.8 ± 1.0
PW	65.7 ± 2.0	46.8 ± 2.0	1.2 ± 0.0	13.2 ± 0.0
BH	61.3 ± 1.0	57.3 ± 2.0	1.4 ± 0.0	9.0 ± 0.0
***Escherichia* sp. OPWW**				
TSB	65.3 ± 1.0	56.6 ± 3.0	1.0 ± 0.0	13.3 ± 1.0
NB	65.9 ± 1.0	57.6 ± 5.0	1.6 ± 0.0	14.1 ± 0.0
PW	63.1 ± 1.0	54.5 ± 2.0	1.6 ± 0.0	11.3 ± 0.0
BH	60.0 ± 2.0	48.8 ± 2.0	2.0 ± 0.0	10.5 ± 2.0
***Corynebacterium* sp. DP**				
TSB	62.2 ± 1.0	59.3 ± 2.0	0.8 ± 0.0	11.8 ± 2.0
NB	62.6 ± 1.0	56.2 ± 3.0	2.0 ± 0.0	13.8 ± 0.0
PW	59.1 ± 1.0	56.5 ± 2.0	1.4 ± 0.0	10.1 ± 1.0
BH	51.2 ± 1.0	51.9 ± 2.0	1.6 ± 0.0	14.7 ± 1.0

Legend: BATH expressed as the percentage of bacteria that partition into 0.5 ml of *n*-octane/*p*-xylene. SAT indicates lowest of concentration M $(NH_4)_2SO_4$ in the reaction mixture that produced visual clumping. Uptake of Congo red dye greater than 10µg was scored as strongly hydrophobic. TBS: Tryptone soy broth, NB: Nutrient broth, PW: Peptone water, BH: Bushnell Haas broth.

Conclusion

In conclusion, the investigation showed that among the four growth media used in the study TBS was most suitable in expression of hydrophobicity of refinery effluent bacteria. This was followed by NB and PW while BH was the least medium for expression of hydrophobicity in effluent bacteria. This also shows that cell surface hydrophobicity of refinery effluent organisms can be enhanced by growing the organisms in TBS medium in order to enhance their bioremediation capacity as hydrophobicity is correlated with biodegradation.

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