Influence of Growth Media Composition on the Emulsifying Activity of Bioemulsifiers Produced by Four Bacterial Isolates with Wide Substrate Specificity

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Abstract: The influence of growth media composition on the emulsifying activity of bioemulsifiers produced by four bacterial isolates were monitored by using three standard growth media formulations of Rosenberg *et al*, 1979, Monticallo *et al*, 1985 and Mills *et al*, 1978 with a common carbon source, Acetate but with varied Nitrogen, Phosphate and Trace element constituents. Three of the four bacterial isolates namely; *Pseudomonas mallei, Pseudomonas pseudomallei* and *Pseudomonas* sp. recorded their highest emulsifying activity of 30.80, 26.30 and 29.10 µ/ml respectively when grown on Rosenberg *et al*, 1979 growth medium with respective pH optimums of 7.09, 7.60 and 7.40 while the last isolate, *Pseudomonas aeruginosa* recorded its highest emulsifying activity of 28.40 µ/ml when grown on Mills *et al*, 1978 growth medium with an optimum pH of 8.11. Monticello *et al*, 1985 growth medium which lack $MgS0_4$ salts recorded lower emulsifying activity in all the four bacterial isolates tested. The results indicate that the three bacterial isolates that grew better on Rosenberg *et al*, 1979 growth medium showed preference for $(NH_4)_2S0_4$ as opposed to $NaN0_3$ as the ideal source of Nitrogen, however the reverse was the case with the isolate that grew better on Mills *et al*, 1978 growth medium which showed preference for $NaN0_3$ as its best Nitrogen source. Magnesium ions and other trace elements constituents of Rosenberg *et al*, 1979 growth medium are suspected to stimulate higher emulsifying activity as the other growth media formulations that lacked them showed lower emulsifying activity.
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1. Introduction

Bioemulsifiers are amphipathic molecules which can be divided into two major groups; (i) Low molecular weight compounds such as glycolipids and phospholipids which lower interfacial tension between hydrophobic liquids and water and thus reduce the energy required for emulsions. (ii) Polymers which stabilise emulsions (Rosenberg, 1986). In the recent years, bioemulsifiers have received increasing attention because of their role in the growth of microorganisms on water insoluble hydrophobic materials such as hydrocarbons and also because of their commercial potential in the cosmetics, food and agricultural industries (Rosenberg, 1986).

The effect of variations of media components such as carbon, nitrogen, phosphate and metal ions on bioemulsifier production have been investigated by several authors. These investigations revealed that different types of microorganisms have preference for different and specific media components for optimum production of bioemulsifiers.

Carbon is a very essential component of media for microbial growth and different microorganisms that produce bioemulsifier have preference for specific sources of Carbon for optimum production of their bioemulsifier. Navon-Venezia *et al*, 1995 demonstrated that the biological activity of a bioemulsifier, Alasan produced by *Acinetobacter radioresistens* was higher when Citrate was used as carbon source than when Acetate or Tris-Hcl buffer was used. With different bacterial isolates, *Pseudomonas mallei* and *Pseudomonas pseudomallei*, Okoro *et al*, 2002 advanced that combination of acetate and diesel seem to be the preferred carbon sources when compared with other carbon sources such as Crude oil, Olive oil, Kerosine and diesel for optimum bioemulcifier production by these organisms.

Nitrogen sources also effect the production of bioemulcifier by microorganisms. Among the inorganic salts tested by Desai and Banat, 1997, Amonium salts and Urea were the preferred nitrogen sources for optimum bioemulsifier production by *Athrobacter paraffineus.* Nechemania and Rosenberg (1983) have also demonstrated the preference of Amonium ions as nitrogen sources in the production of bioemulsifier by *Acinetobacter calcoaceticus* strains. Other authors such as Okoro *et al*, 2002, Moussa *et al*, 2006, Namir *et al*, 2009 and Batista *et al*, 2010 have equally demonstrated maximum production of bioemulsifier when ammonium ions were used as nitrogen sources. On the contrary, other

investigations carried out by Navon-Venezia *et al*, 1995 showed that Urea was the best nitrogen source for the production of a bioemulsifier, Alasan by *Acinetobacter radioresistens*. Similarly, Sifour *et al*, 2005 also demonstrated that Bacillus species showed preference for Urea as the ideal nitrogen source for bioemulsifier production. On the contrary, some other researchers have implicated nitrate as the best nitrogen source for bioemulsifier production. Graziella *et al*, 2010 demonstrated that nitrate was the best nitrogen source for production of bioemulsifier by *Rhodococcus erythropolis*. Guera-Santos *et al*, 1984 also demonstrated the preference of nitrate as an ideal nitrogen source for bioemulsifier production by *Pseudomonas aeruginosa*, same goes with Ramana and Karanth (1989) who advanced that *Pseudomonas aeruginosa* showed more preference for $NaN0_3$ as the best nitrogen source in the production of glycolipid under submerged conditions.

Other media constituents like Phosphate and metal ions also influence the production of bioemulsifier by microorganisms. For instance, in the production of glycolipid bioemulsifier by *Pseudomonas aeruginosa*, it was discovered that using K_2HP0_4 gave 3 fold yield of glycolipid than what was obtained when KH_2P0_4 was used (Ramana and Karanth, 1989). Magnesium ions have also been shown to positively affect the process of emulsification (Sifour *et al*, 2005). Navon-Venezia *et al*, 1995 also demonstrated that Magnesium ions stimulated the activity of Alasan produced by *Acinetobacter radioresistens* over a wide range of pH.

In summary, both past and present investigations relating to the influence of media constituents on bioemulsifier production showed that different microorganisms have preference for different and specific sources of carbon, nitrogen, phosphate and metal ions for optimum production of bioemulsifier. The emulsifying activity of the bioemulsifier produced is very important because it determines to a great extent the strength and quality of the bioemulsifier produced.

In the present study, we investigated the influence of growth media composition on the emulsifying activity of the bioemulsifier produced by four bacterial isolates with wide substrate specificity. Three standard media formulations of Rosenberg *et al*, 1979, Monticallo *et al*, 1985 and Mills *et al*, 1978 were used in this study for the purpose of comparison. The common carbon source used in all the three media was Acetate but sources of nitrogen, phosphate and metal ions varied. We conducted preliminary characterisation of the bioemulcifier produced by these isolates and their various hydrocarbon substrate specificities which ranged from normal alkanes to aromatics and complex hydrocarbon mixtures.

2. Materials and Methods:

Bacterial Isolates:

The four bacterial isolates used in the study namely *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Pseudomonas aeruginosa and Pseudomonas* sp. were isolated from produced water from Chevron's Escravos tank farm using minimal salts medium of Mills *et al*, 1978 and partial characterisation was done with the aid of the BBL enterotube computerised identification systems as previously described (Okoro, 1999). These isolates were maintained in nutrient agar slants at low temperature (5^0C) and sub-cultured weekly.

Growth Media Composition:

(i) Rosenberg *et al;* (1979): Composition (g/l): NaCl(5), Na_2HP0_4,(13.7), KH_2PO_4(7.26), $(NH_4)_2SO_4$(3), $MgSO_4$(0.4). Trace elements (mg/10ml); $CaCl_2.2H_20$(3.68), $CuS0_4.5H_20$(6.24), $FeS0_4.7H_20$(5.94), $MnS0_4.2H_20$(4.22), $ZnS0_4.7H_20$(7.88), $CoCl_2.6H_20$(6.96).

(ii) Monticello *et al;* (1985): Composition (g/l): KH_2P0_4(4), Na_2HP0_4(4), $(NH_4)_2S0_4$(2), $CaCl_2.2H_20$(0.001), $FeS0_4.7H_20$(0.001).

(iii) Mills *et al*; (1978): Composition (g/l): NaCl(10), KCL(0.29), $MgS0_4.7H_20$(0.42), KH_2P0_4(0.83), Na_2HP0_4(1.25), $NaN0_3$(0.42).

Hydrocarbon substrate specificity of the four bacterial isolates:

The ability of the four bacterial isolates to grow on pure hydrocarbon substrates as sole carbon source were tested on a liquid minimal salts media of Mills *et al*, 1978. All the substrates except the highly flammable ones were autoclaved before use, the flammable ones such as n-alkanes, and kerosene were sterilised by filtration before use. 100mls of the minimal salt media was prepared in a 250ml Erlenmeyer flask and 0.1% hydrocarbon substrate was inoculated followed by the addition of 1ml of the bacterial inoculum from the already prepared nutrient broth and incubation for 48hrs at room temperature. Emulsion turbidity was measures as described in Rosenberg *et al*, 1979.

Determination of Emulsification activity:

The standard emulsification assay of Rosenberg *et al*, 1979 was used in the determination of emulsification activity of the four bacterial cultures used for the studies. The samples to be tested (0.5-0.1ml) were introduced into a 125ml flask containing TM buffer (20Nm Tris-HCL) pH (7.0), 10nM, $MgS0_4$ to a final volume of 7.5ml and then 0.1ml of a 1:1 (v/v) mixture hexadecane and 2-

methylnaphthalene was added. The samples were incubated at 30^0C with reciprocal shaking (160 strokes/min) for 1hr. Turbidity was then determined in a Klett-Summerson photometer (fitted with green filter). One unit of emulsifying activity per millilitre is defined as the amount of biopolymer that yielded 100 Klett units in the assay mixture. Emulsion turbidity was directly proportional to the amount of biopolymer produced.

Partial Biochemical characterisation of Bioemulsifiers;

Lipid Analysis: Thin layer chromatography was carried out on a 20 by 20 cm precoated silica gel plates with petroleum ether, diethyether and acetic acid (90:10:1) as developing solvents. After air drying, the silica gel plates were stained with 5% sulphuric acid in 95% ethanol followed by heating at 15^00C for 30mins. RF values of developed spots were calculated and compared with values of standard compounds in similar solvents as described by Kates (1972).

Protein Analysis: The protein content of the cell extracts was determined by using the method of Bradford (1976). The reagent contained Coomassie blue, 9250 (0.16ml), Percloric acid (5.15ml), and distilled water added to make up to 200ml. The reagent was stirred in a dark bottle overnight and filtered with a Whatman No. 1 filter paper. Protein solution (0.5ml) was added to a 1ml cuvette and 0.5ml of the reagent was added. The absorbance at 620nm was red immediately against a reagent blank made up of 0.5ml of water and Coomassie reagent. The concentration of the protein was extrapolated from the standard curve prepared with bovine serum albumen as a standard.

Carbohydrate Analysis; The carbohydrate content of the bioemulsifier was estimated by using Anthrone method as described by Spirro (1966). 720ml of concentrated sulphuric acid was added to 280ml of distilled water. This was followed by the addition of 500mg of anthrone and 10g of thiourea and mixing till the contents were properly dissolved. The reagent was cooled by storing in a refrigerator 24hrs. before use. A standard curve was prepared by adding 20-200µg of glucose in 1ml of water in pyrex tubes. The test reaction was carried out by adding 1ml of the bioemulsifier extract to 5mls of cold anthrone reagent in a 10ml testube. The tubes were shaken vigorously to ensure complete mixing and this was followed by capping and heating the tubes in a boiling water bath for 15mins and cooling thereafter. The absorbance was red at 620nm against a reagent blank.

SDS-Polyacrylamide Gel Electrophoresis: Polyacrylamide gel (12%) electrophoresis was carried out to determine the molecular weight of proteins as described in Bradford (1976). The following protein makers purchased from sigma chemicals (Sweden) were used as standard makers in the electrophoresis. They include Lysosyme, egg white (14,000Da), β-Lactoglobulin ,Bovine milk(18,400Da), Trypsinogen, Bovine pancreas (24,000DA), Pepsin, Porcine stomach mucosa (34,700Da), Egg albumin (45,000Da), Bovine plasma albumin (66,000Da)

3. Results:

Hydrocarbon substrate specificity of the bioemulsifier produced by four bacterial isolates

Various hydrocarbon substrates were tested on the bioemulsifier produced by the four bacterial isolates to determine their emulsion turbidity. All the four bioemulsifiers tested exhibited very wide substrate specificity ranging from n-Alkanes to aromatics and some complex hydrocarbon mixtures such as Hexadecane+ Methylnaphthalene (1:1), Benzene + Cyclohexane (1:1), Toluene + Cyclohexane (1:1), Olive oil, Kerosine, Diesel oil and Crude oil. Among all the hydrocarbon substrates tested, the highest emulsion turbidity was recorded with crude oil, and closely followed by diesel oil. Lower molecular weight hydrocarbons (n-Alkanes) recorded lower emulsion turbidities than mixtures of complex hydrocarbons. The results are shown in Table 1.

Effects of media constituents on the growth and activity of the bioemulsifier producing bacterial isolates;

The three growth media used in the study include;

1. Rosenberg *et al*, 1979 growth media with the following composition; NaCl (5), Na_2HP0_4,(13.7), KH_2PO_4 (7.26),($NH_4)_2SO_4$ (3), $MgSO_4$ (0.4), Trace elements (mg/10ml); $CaCl_2.2H_20$(3.68), $CuS0_4.5H_20$(6.24), $FeS0_4.7H_20$(5.94), $MnS0_4.2H_20$(4.22), $ZnS0_4.7H_20$(7.88), $CoCl_2.6H_20$(6.96).
2. Monticello *et al*; (1985) growth medium with the following composition; KH_2P0_4(4), Na_2HP0_4(4), $(NH_4)_2S0_4$(2), $CaCl_2.2H_20$(0.001), $FeS0_4.7H_20$(0.001).
3. Mills et al; (1978) growth medium with the following composition; NaCl(10), KCL(0.29), $MgS0_4.7H_20$(0.42), KH_2P0_4(0.83), Na_2HP0_4(1.25), $NaN0_3$(0.42).

Table 1: Hydrocarbon Substrate Specificity of the Bioemulsifier produced by the bacterial isolates used in the present study

Hydrocarbon Substrates	**Emulsion Turbidity (KU) of the Bacterial Cultures**			
ALKANES	***Pseudomonas mallei***	***Pseudomonas pseudomallei***	***Pseudomonas aeruginosa***	***Pseudomonas sp.***
n-Pentane	36	44	56	22
n-Hexane	28	14	32	43
Cyclohexane	12	32	44	32
Decane	22	46	13	38
Pentadecane	110	86	65	120
Hexadecane	88	25	63	65
Octadecane	147	102	132	160
AROMATICS				
Benzene	144	186	88	67
Toluene	22	12	67	88
Xylene	36	82	96	120
Butyl benzene	46	48	75	87
Octyl benzene	76	81	66	43
HYDROCARBON MIXTURES				
Hexadecane+ Methylnaphthalene (1:1)	220	160	180	107
Benzene + Cyclohexane (1:1)	140	260	87	170
Toluene + Cyclohexane (1:1)	33	48	13	66
Olive OIL	320	420	330	440
Kerosine	110	46	260	28
Diesel Oil	440	360	380	280
Crude Oil	760	650	580	460

All the three growth media used for this study have a combination of KH_2P0_4 and Na_2HP0_4 as the source of phosphate. The Nitrogen source however differed, while Rosenberg *et al,* 1979 and Monticello *et al* 1985 media had ($NH_4)_2SO_4$ as their nitrogen source, Mills et al; had $NaN0_3$ as its nitrogen source. Magnesium sulphate salts which is essential for enhancement of emulsification was present in both Rosenberg *et al,* 1979 and Mills et al;1978 growth media but absent in Monticello *et al*; (1985) growth medium. Rosenberg *et al,* 1979 and Mills et al; (1978) growth media used NaCl salts while Monticello *et al*; (1985) growth medium used $CaCl_2$ salts. Rosenberg *et al,* 1979 growth media in addition fortified its growth media with some trace elements such as (mg/10ml); $CaCl_2.2H_20$(3.68), $CuS0_4.5H_20$(6.24),$FeS0_4.7H_20$(5.94),$MnS0_4.2H_20$(4.22), $ZnS0_4.7H_20$(7.88), $CoCl_2.6H_20$(6.96). All these media components in one way or the other influenced the emulsification activity of the bioemulsifier produced by the four bacterial isolates.

The emulsification activity of the bioemulsifier produced by the four bacterial isolates showed that *Pseudomonas mallei, Pseudomonas pseudomallei and Pseudomonas sp.* all grew better in Rosenberg *et al,* 1979 growth medium and the emulsification activity of the bioemulsifier produced were 30.80, 26.30 and 29.10 µ/ml respectively with respective final pH values of 7.09, 7.11 and 7.40. The fourth organism *Pseudomonas aeruginosa* however grew better in Mills *et al*, 1978 growth medium and the bioemulsifier produced had an emulsification activity of 28.40 µ/ml at a final pH of 8.11. The detailed results are shown in Table 2.

Partial biochemical characterisation of the bioemulsifiers produced by the four bacterial isolates

Partial biochemical characterisation of the bioemulsifiers produced by the four bacterial isolates showed that *Pseudomonas mallei* and *Pseudomonas pseudomallei* showed presence of carbohydrate and protein moieties with no trace of lipids. The respective concentrations of proteins and carbohydrates of the bioemulsifier produced by *Pseudomonas mallei* were 20.77 and 413g/L while that of *Pseudomonas pseudomallei* were 27.23 and 242g/L. The protein moieties of the two bacterial isolates had a molecular weight of about 34,700Da suggesting that the two bacterial isolates might be closely related. They were tentatively classified as glycoproteins.

Table 2: Emulsification Activity of the Bioemulsifier Producing Bacterial Isolates Using Different Growth Media

BACTERIAL CULTURES	GROWTH MEDIA	Final pH	Turbidity (A_{600})	Activity (µ/ml)
Pseudomonas mallei	*Rosenberg et al; (1979)*	7.09	1.42	30.80
	Monticello et al; (1985)	7.25	0.88	27
	Mills et al; (1978)	8.71	0.63	7.20
Pseudomonas pseudomallei	*Rosenberg et al; (1979)*	7.11	0.99	26.30
	Monticello et al; (1985)	7.60	0.68	14.10
	Mills et al; (1978)	8.54	0.64	1.90
Pseudomonas aeruginosa.	*Rosenberg et al; (1979)*	7.11	1.32	23.60
	Monticello et al; (1985)	7.20	1.40	26.80
	Mills et al; (1978)	8.11	1.60	28.40
Pseudomonas sp.	*Rosenberg et al; (1979)*	7.40	1.38	29.10
	Monticello et al; (1985)	7.80	1.23	16.60
	Mills et al; (1978)	8.12	1.30	21.10

The two other bacterial isolates; *Pseudomonas aeruginosa* and *Pseudomonas* sp. produced bioemulsifiers with considerable concentrations of carbohydrate and lipid moieties but no trace of protein. The respective concentrations of carbohydrates and lipids in the two bacterial isolates were 312:12.6g/L and 286:16.8g/L. The bioemulsifier produced by the two bacterial isolates were tentatively classified as glycolipids.

4. Discussion

The data presented in this paper showed that media constituents can influence the emulsification activity of the bioemulsifier producing bacterial isolates used in the study. With Rosenberg *et al*, 1979 growth media, *P. mallei*, *P. pseudomallei* and *Pseudomonas* sp. grew better in it than the other two and the bioemulsifier produced had an emulsification activity of 30.8, 26.30 and 29.10 µ/ml respectively. Comparatively, the same three group of bacterial isolates grew poorly on Monticello *et al*, 1985 growth medium with respective emulsification activity values of 27, 14.10 and 16.60. It should be noted that both Rosenberg *et al*, 1979 growth medium and Monticello *et al;* (1985) growth medium had a common nitrogen source $(NH_4)_2S0_4$ but differed in the presence of magnesium salts and other essential trace elements which were present in Rosenberg *et al*, 1979 growth medium but lacking in Monticello *et al*; (1985)growth medium. The presence of magnesium salts and other essential trace elements in Rosenberg *et al*, 1979 growth medium must have contributed in the enhancement of the emulsification activity of the bioemulsifier produced. Some investigators like Sifour *et al*, 2005 and Batista *et al*, 2010 have demonstrated that magnesium salts and other trace elements positively affect the emulsification activity process.

On the contrary, *Pseudomonas aeruginosa* grew better on Mills *et al*, 1978 medium than in Rosenberg *et al*, 1979 and Monticello *et al*; (1985)growth media. It should be noted that the basic difference between the three growth media under investigation was the source of nitrogen. Whereas Mills *et al*, 1978 growth medium uses nitrate as its nitrogen source, Rosenberg *et al*, 1979 and Monticello *et al*; (1985) growth media uses $(NH_4)_2S0_4$ as their preferred nitrogen source. Despite the fortification of Rosenberg media with trace elements, *Pseudomonas aeruginosa* still preferred nitrate as its ideal nitrogen source for optimal production of its bioemulsifier. Many investigators have reported that *Pseudomonas aeruginosa* have always shown preference for nitrate as its best nitrogen source (Ramana and Karanth, 1989, MacElwee and Treros, 1990, Guero-Santos *et al*, 1984, and Robert *et a*l, 1989). All the other bacterial isolates that grew better on Rosernberg *et al*, 1989 growth medium however showed preference for $(NH_4)_2S0_4$ salts as opposed to $NaN0_3$ as the preferred nitrogen source.

Partial biochemical characterisation of the bioemulsifier produced by the four bacterial isolates showed that *P. mallei* and *P. pseudomallei* produced the glycoprotein type of bioemulsifier while *P. aeruginosa* and *Pseudomonas* sp. produced the glycolipid type. The glycoprotein bioemulsifier produced by *P. mallei* and *P. pseudomallei* though have not been reported widely in literature are very potent and have been used in the past to enhance the remediation of hydrocarbon contaminated mangrove swamp in the Nigerian oil rich Niger Delta (Okoro, 2009). The glycolopids produced by *P. aeruginosa* and *Pseudomonas* sp. are one of the commonest type of bioemulsifier and have been reported widely in literature (Javis and Johnson, 1949, Hisatsukka *et al*,

1971, Itoh and Suzuki, 1972, Ramana and Karanth, 1989 and Guerra Santos *et al*, 1984.

5. Conclusion

In conclusion, the present study have clearly demonstrated that some essential components of growth media have specific influence on the emulsification activity of the bioemulsifier produced by the four bacterial isolates used in the present study, while some components of the media depending on the type of bacterial isolate influenced bioemulsifier production and its emulsification activity positively, the reverse was the case with other media components. The present study have clearly established that magnesium salts and other essential trace elements in Rosenberg *et al*, 1979 growth media influenced the emulsification activity positively. *Pseudomonas aeruginosa* also proved its consistency on the use of nitrate as its best nitrogen source.

In summary, all the bioemulsifier produced by the four bacterial isolates used in the present study had very wide substrate specificity. They emulsified considerably a variety of water in oil emulsions, including n-Alkanes, aromatics, complex hydrocarbon mixtures, crude oil, olive oil, diesel oil and kerosene. With the ideal media components, the emulsification activity of the produced bioemulsifier can be further enhanced for commercial application.

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