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GC-MS profile of Biosurfactant producing and Hydrocarbon degrading *P. aeruginosa* NGB4 in liquid culture system

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ABSTRACT

Aim: The ability of biosurfactant and hydrocarbon degrading capability of the *P. aeruginosa* NGB4 has been evident by GC -MS profiling. **Materials and Methods**: Industrial waste water supplemented with soybean oil, *P. aeruginosa* CFU set at 2 x 10^8 per ml has been tested for the formation of biosurfactant and metabolites under laboratory studies in liquid culture systems and further identified by GC-MS analysis. **Results and Discussion**: The ability of *P. aeruginosa* NGB4 to form biosurfactant found to enhance the degradation capability of the waste water which has resulted in faster degradation and confirmed by number of intermediate metabolites formed. **Conclusion**: Potential feature of *P. aeruginosa* NGB4 for hydrocarbon degradation by virtue of biosurfactant production has been elucidated which could be utilized in waste water hydrocarbon degradation.

KEYWORDS: Biosurfactant; Hydrocarbon degradation; *P. aeruginosa;* GC-MS.

1. INTRODUCTION

The ability of *P. aeruginosa* to produce biosurfactant has been well established in recent studies. This feature makes them efficient source for biodegradation of hydrocarbon and they can utilize different waste sources to produce biosurfactant as well as to biotransform them as low molecular weight moieties. Olive mill waste (OMW) which has been one of the pollutants and tough to degrade has been reported to be the substrate on which *P. aeruginosa* can produce maximum of biosurfactant. At 2% w/v of OMW in the medium, *P. aeruginosa* produced 8.78 mg/L biosurfactant, while it has been increased many fold when the OMW percent reaches to 10% and found to produce 191.46 mg/L biosurfactant.[1]

P. aeruginosa JP-11 isolated from marine coastal sediments of Odisha, India showcased to utilize 99% of the biphenyl within 72 h. Based on the technologies, formed intermediate such as 2-Hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate has been identified. Assimilation of biphenyl was initiated by its deoxygenation, forming cis-2, 3-dihydro-2, 3-dihydroxybiphenyl subsequently transformed to 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate. In lower pathway cis-1,6-dihydroxy-2, 4-cyclohexadiene-1-carboxylic acid was detected which formed catechol before entering into the Krebs cycle. To these metabolites formed in the process, it was linked with the enzyme catechol-1, 2-dioxygenase in the cell-free extract of *P. aeruginosa* JP-11. Also genes like, biphenyl dioxygenase encoded by bphA gene along with biosurfactant rhamnolipid synthesizing gene rhlAB was also amplified. The study showcased that rhlAB increase in expression upto 258 folds once exposed to high biphenyl stress.[2] *P. aeruginosa* DSVP20 found to be positive for the glycolipid biosurfactant production at a rate of 6.7 g/L after 72 h at 150 rpm and at a temperature of 30°C. Further it can degrade eicosane (97%), pristane (75%), and fluoranthene (47%) put up their potential role in bioremediation of petroleum hydrocarbon-contaminated soil.[3] In the present study, an attempt has been made to elucidate the GC-Ms profile of the degraded compounds and biosurfactant produced by the *P. aeruginosa* when treated in presence of waste water.

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2. MATERIALS AND METHODS

2.1. Isolation and Identification of P. aeruginosa

The sample of waste water collected from the Kosmi industrial area identified for the bacterial isolate with biosurfactant production and then identity as *P. aeruginosa* NGB4 strain confirmed by the oil spreading technique[4]; Emulsification Activity Measurement[5] and by involving 16S rRNA gene sequencing with assigned accession number LC176072.

2.2. Detection of Biosurfactant and metabolites during degradation

As the ability of *P. aeruginosa* NGB4 has been confirmed for biosurfactant production, its involvement in the degradation of industrial waste water supplemented with soybean oil has been investigated by involving the GC-MS technique. In the process, 100 ml of 0.1X M9 medium was supplemented with sterilized 20% waste water effluent coming from soybean processing plant and kept in 250 ml. The flask was inoculated with *Ps. aeruginosa* NGB4 in a CFU set at 2 x 10^8 per ml. In a control, medium kept uninoculated without any culture. The entire flasks were aerated with sterile air under controlled room temperature of 28^{O}C and samples were withdrawn for GC MS analysis of 4th day, 9th day Soybean oil, waste water and the developed foam inside of the flask which was indicator of the biosurfactant production and all sets were compared for available metabolites and biosfurfactants.

3. RESULTS

3.1. Detection of Biosurfactant and Metabolites by GC-MS technology

Once the biosurfactant production ability of the *P. aeruginosa* NGB4 has been evidenced, attempt has been made to detect the particular biosurfactant expressed by the isolate. In this study comparative change in nature of active compound in every stage of the process has been investigated. In an attempt first the content of soybean oil has been investigated by GC MS method and about 14 compounds have been detected named as (1) (-)-Myrtenyl acetate, (2) Caryophyllene, (3) .gamma.-Elemene, (4) Spiro[4.5]dec-7-ene, 1,8-dimethyl-4-(1-m, (5) 6-Heptadecyne, 1-chloro-, (6)Phenol, 2,4,6-tris(1-methylethyl)-, (7) Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-, (8) Naphthalene, 1,2,3,4-tetrahydro-1,6-dime Isoledene, (9) 2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahy, (10) Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-d Diepi-.alpha.-cedrene epoxide, (11) Octadecane, 1-(ethenyloxy)-, (12)Agarospirol, (13) 4-Carbomethoxy-4-[2-(2-carbomethoxyvinyl, (14) Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-Ethanol, 2-(9,12-octadecadienyloxy)-, (Z) .

Based on the GC-MS results the water of the Kosmi dam contaminated with the soybean oil and other pollutant when identified for the compounds firstly following aromatic and aliphatic compounds were detected before any treatment by NGB4 isolate and were recognized as : (A) 1,2-Bis(p-acetoxyphenyl)ethanedione, (B) Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyc, (C) 1,3-Cyclohexadiene-1-carboxylic acid, 2, (D) Benzestrol, (E) 4-Methyl-2-tert-octylphenol, (F) 4,6-Bis(4-ethoxybenzylthio)-5-nitropyrim, (G) Tricyclo[4.3.1.1(3,8)]undecane-3-carboxy, (H) 4-Butoxy-2-hydroxybenzonitrile, (I) Bicyclo[4.1.0] hepta-2,4-diene, 2,3,4,5-t, (J) Benzyloxy(butyl)dimethylsilane, (K) 5,6,7-Trimethoxy-1-indanone, (L) 2-[3-(4-tert-Butyl-phenoxy)-2-hydroxy-pr, (M) 2,5,7,10-Tetraoxa-6-silaundecane, 6-(2-m, (N) Benzoic acid, 2,4-bis[(trimethylsilyl)ox, (O) Benzimidazol-2(3H)-one, 5-(2,2,2-trichlo and (P) 4-(4-Oxo-1,2,3,4,6,7,12,12b-octahydropyr.

Further when the NGB4 allowed to grow in presence of these chemicals available in the waste water in early 48 hours high amount of froth was produced probably because NGB4 released biosurfactant and as obvious such foam formation was not evidenced in a control flask. The foam sampled from the flask was also subjected to the biosurfactant detection by GC MS and overall twenty six compounds were detected from those such as (A) 2-Azido-2,4,4,6,6-pentamethylheptane, (B) 1R,4S,7S,11R-2,2,4,8-Tetramethyltricyclo, (C) .tau.-Muurolol, (D) 1,3-Benzodioxole, 5-(2-propenyl)-, (E) Benzene, 1,2-dimethoxy-4-(2-propenyl)-, (F) Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-, (G) Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-d, (H) Tritetracontane, (I) 1-Hentetracontanol, (J) Tridecanoic acid, 12-methyl-, methyl est, (K) Benz[a]azulene, (L) Benzene, 1-methyl-3,5-bis[(trimethylsily, (M) 1-Decanol, 2-hexyl-, (N) 2-Butenal, 2-methyl-4-(2,6,6-trimethyl-1, (O) 4,5,6,7-Tetrahydroxy-1,8,8,9-tetramethyl, (P) Octadecanoic acid, 3-hydroxy-2-tetradecy, (Q) Cyclopropanenonanoic acid, 2-[(2-butylcy, (R) l-(+)-Ascorbic acid 2,6-dihexadecanoate, (S) Fluoranthene, (T) 13-Hexyloxacyclotridec-10-en-2-one, (U) 9-Octadecenoic acid (Z)-, 2,3-dihydroxyp, (V) Estra-1,3,5(10)-trien-17-one, 3-methoxy-, (W) 1-Dodecanol, 3,7,11-trimethyl-, (X) 1-Hentetracontanol, (Y) 1-Hexacosene, (Z) 17-Pentatriacontene.

In progress, the reaction was allowed to continue so that the degradation of the hydrocarbon could be carried out in due presence of biosurfactant and on 4th day first sample of the reactor was collected and analyzed for compound detection. Surprisingly only five compounds were identified as (A) Phenol, 2,4-bis(1,1-dimethylethyl)-, (B) Pentadecanoic acid, 13-methyl-, methyl e, (C) 2,8,9-Trioxa-5-aza-1-silabicyclo[3.3.3]u, (D) Spiro[3-cyclohexene-1,2'-[2H]furo[3,2-f], and (E) (2-Methoxy-1,3,2-thiozin)[5,10,9]androst; was detected but in increasingly high concentration which suggested that these may be the end or intermediate products of the reaction carried out by the NGB4 activity.

On 9th day also sample analyzed by GC MS in which 46 compounds were detected as (1)Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro, (2)Agarospirol, (3) 1R,4S,7S,11R-2,2,4,8-Tetramethyltricyclo, (4) Cadala-1(10),3,8-triene, (5) Strophanthidol, 3,19-diacetate, (6) Cyclohexane, 1,1,3-trimethyl-2,3-epoxy-2, (7) 7-Tetracyclo[6.2.1.0(3.8)0(3.9)]undecano, (8) Aristolene epoxide, (9) 2,4,6-Trimethylmandelic acid, (10) 3Beta-

acetoxy-4,4,8,10,14-pentamethyl-17, (11) Phenol, 2,4-bis(1,1-dimethylethyl)-, (12) 1,5-Diphenyl-2H-1,2,4-triazoline-3-thion, (13) 2-Propenoic acid, 3-(3,4-dimethoxyphenyl, (14) 6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7, (15) Caryophyllene-(I1), (16) 4,6,10,10-Tetramethyl-5-oxatricyclo[4.4., (17) Benzene, (1-pentylheptyl)-, (18) 2H-Cyclopropa[g]benzofuran, 4,5,5a,6,6a, (19) 3-(1-Acetyl-2,2-dimethyl-5-oxocyclopenty, (20) Tritetracontane, (21).gamma.-Gurjunenepoxide-(2), (22) 1,2-Benzenedicarboxylic acid, butyl 2-me, (23) 6S-2,3,8,8-Tetramethyltricyclo[5.2.2.0(1), (24) 1-Hexacosene, (25) 13-Tetradecen-1-ol acetate, (26) 1-Hentetracontanol, (27) 1-Cyclohexyldimethylsilyloxyoctadecane, (28) 6,7-Diphenyl-7H-pyrrolo[2,3-b]pyrazine, (29) Tritetracontane, (30) 1,2-Benzenedicarboxylic acid, butyl cycl, (31) 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6, (32) Pentadecanoic acid, 14-methyl-, methyl e, (33) Bicyclo[9.3.1]pentadeca-3,7-dien-12-ol, (34) 1-(4-Butoxy-2,6-dimethylphenyl)ethanone, (35) Tetrapentacontane, 1,54-dibromo-, (36) 9-Hexacosene, (37) Falcarinol, (38) 2,3-Dihydroxypropyl elaidate, (39) Retinal, 9-cis-, (40) Heptadecanoic acid, 15-methyl-, methyl e, (41) Fenretinide, (42) Hexadecanoic acid, octadecyl ester, (43) 1-Pentacosanol, (44) Aspidospermidin-17-ol, 1-acetyl-19,21-ep, (45) 1-Hexacosene and (46) Phenol, 2,4-bis(1-phenylethyl) having lower molecular weight than earlier metabolites suggested their degradation.

Further during the bioreaction of NGB4, foam has been formed which has showed number of compounds which completely different from the liquid medium compounds recorded in 4th and 9th day indicated that these compounds play a different role in the action which needs to be investigated in greater detail.

During reaction by NBG4 as the waste water hydrocarbons were degraded at a fastest rate as evidenced from the reduction in COD rate (data not shown) several of these compounds must be of biosurfactant nature which has been investigated in the data given in discussion.

4. DISCUSSION AND CONCLUSION

In present study biousrfactant produced by *P. aeruginosa*NGB4 was detected as per GCMS analysis for 4th, 9th day and also foam formed during process along with soybean oil and waste water. On 4thday it has been observed that only five compounds were detected; In which a compound Pentadecanoic acid, 13-methyl-, methyl was found to biosurfactant as per the workers Adekunle AT et al. (2015) where they have identified this compound by GC-MS analysis from *A. niger* and recorded to be biosurfactant.[6] In another study Pentadecanoic acid, 13-methyl-, methyl as glycolipid biosurfactant has been isolated from *P. aeruginosa* only and this evidence confirmed that in genus *Pseudomonas* this compound expresses effectively as a biosurfactant as in accord with study also where strain NGB4 expressed the same on 4th day. Another compound Phenol, 2,4-bis(1,1-dimethylethyl)- has been recognized to be encoded by *P. aeruginosa* NGB4 and probably having anti quorum sensing activity as per data published by Padmavathi AR et al (2014) because this compound act against *S. marcescens* by reducing protease (42%), haemolysin (70%) lipase (84%), prodigiosin (85%) biofilm (85%) activity when tested *in vitro* when purified from marine bacteria.[7]

On 9th day in total 46 active compounds were detected by the GC MS in which agarospirol was recognized as sesquiterpenes and found to be involved as biosurfactant characteristics although no clear evidence was related with the compound.[8-12] Another compound identified as Phenol, 2,4-bis(1,1-dimethylethyl)- also known as (2, 4-Di-tert-butylphenol or DTBP) was recognized as a potent antioxidant and has the ability to modify extracellular polymeric substances of bacteria. In another study DTBP mentioned to inhibit fungal growth and also efficient in anti-biofilm activity. It is also effective in inhibition of hemolysins, phospholipase and aspartyl proteinase which are the crucial virulence factors required for the invasion of *C. albicans*.[7] Another compound tritetracontane has been detected for the first time in any bacterium (in *P. aeruginosa* NGB4) as earlier it has been only isolated from the plant *Dichrocephala integrifolia.*[13] Presence of .gamma.-Gurjunenepoxide-(2) has also been detected for the first time by any bacteria as per literature survey and it has been reported to be present in the essential oils of *Beilschmiedia glabra*.[14]

Since, present study dealt in the degradation of waste water containing unknown hydrocarbons and due to the action of bacteria, series of intermediates have been formed as evident by one of the compound identified as Tritetracontane. During literature survey it has been observed that the tritetracontane is an intermediate of the 1, 2, 4-trichlorobenzene formed by the action of bacterial enzymes.[15] This evidence highlighted many compounds formed on 9th day might have been the intermediates of compounds degraded since none of them was found to be repeating in early 4th day GCMS samples as well as in soyabean oil or waste water and also recorded to be of low molecular weight. It has been recorded that low molecular weight (604) tritetracontane was also found to be the intermediate of engine and crude oil degradation catalyzed by *Acinetobacter* species and *Bacillus mucilaginosus* respectively which confirms the ability of *P. aeruginosa* strain NGB4 strains also as it has also been recorded with intermediates such as tritetracontane and many others not mentioned as per our investigation.[15,16] Another biosurfactant encoded by *P. aeruginosa NGB4* strain detected by GC MS analysis

as Pentadecanoic acid, 14-methyl-, methyl ester (CAS 5129-60-2) has been found to be important in the degradation as it has also been reported in fungi such as *Aspergillus niger* and *Aspergillus flavus* capable of degrading crude oil.[6] On 9th day of degradation another vital biosurfactant was detected as Heptadecanoic acid, 15-methyl-, methyl ester (mol. wt. 298.50) which was also reported to be co-expressed along with Pentadecanoic acid, 14-methyl-, methyl ester; 10 - Octadecenoic acid, methyl ester; 9,12,15 -Octadecatrienoic acid, (Z,Z,Z)- ; Docosanoic acid, methyl ester $C_{23}H_{46}O_2$ and 13,16-Octadecadiynoic acid,methyl ester in fungus *A. niger* when detected by GC-MS analysis.[6] Eventually, in the present study during and after degradation of hydrocarbons it has been observed that metabolites formed majorly belonged to phytosterols, terpenoids, fatty acids, fatty acid esters, alkyl halides, phenols, alcohols, ethers, alkanes, and alkenes as per GC-MS analysis.

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