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# GREEN FLUORESCENT PROTEIN BASED MICROBIAL BIOSENSOR FOR BIPHENYL DEGRADATION

**Bholay A. D.*, Prajapati Hiren V., Dharaskar Shrikant P. and Wagh Vishal A.**

P.G. Dept. of Microbiology K.T.H.M College, Nashik, M.S, India.
adbholay@gmail.com*, hiren.prajapati91@gmail.com, dharaskar.shri@gmail.com

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## ABSTRACT

Biphenyl is a natural component of coal tar, crude oil and natural gas. It has been widely used in organic synthesis, food preservatives, heat transfer fluids and in the synthesis of polychlorinated biphenyls. It may cause many disorders in humans including cancer. Biphenyl degrading bacteria were isolated from activated sludge sample. Biphenyl degradation ability of all the isolates was determined. One of the isolates showing maximum biphenyl degradation was identified as *Stenotrophomonas maltophilia* by VITEK 2 system. From recombinant *E. coli DH5α* (*E. coli DH5α* transformed using pUC18 cloned with GFP gene) GFP plasmid was isolated and used for transformation of *S. maltophilia* cells. As a result the newly transformed cells having ability to degrade biphenyl were also able to give fluorescence. *E coli DH5α and S. maltophilia* wild showed 24% and 89.9% bipheny degradation respectively. *S. maltophilia transformed cells* showed 97.5% biphenyl degradation which is very high as compared to *E coli DH5α* and *S. maltophilia* wild cells. This ability of the *transformant* was utilised to develop green fluorescence protein based biosensor for biphenyl biodegradation and bioremediation processes. Moreover the *S.maltophilia* has not so far been reported as biphenyl degrader.

**Keywords:** biphenyl, biodegradation, plasmid, transformation, biosensor, *Stenotrophomonas maltophilia.*

## INTRODUCTION

Biphenyl is a natural component of coal tar, crude oil, and natural gas. It has been widely used in organic synthesis, food preservatives, heat transfer fluids, and the synthesis of polychlorinated biphenyl[1]. Although the applications of biphenyl has been decreased in recent years, it still remains in the environment, resulting in serious environmental problems. According to its toxicological properties, biphenyl in the diet has been reported to cause kidney disorders, reduced life span [2] and results in bladder cancer in animals [3] and can also cause slight eye irritation, hepatotoxicity and toxic effects on the central and peripheral nervous systems. The use of microorganisms is expected to be an effective tool for bioremediation of polluted environment. Lunt and Evans 1970 and Catelani *et al*. 1970 first reported the bacterial

isolates that could grow on biphenyl as sole source of carbon and energy. Since then, several biphenyl-degrading bacteria have been isolated, and biphenyl degradation by these microorganisms has been widely studied [4].

An upper pathway via dioxygenation at the 2, 3-position is the major catabolic pathway of biphenyl biodegradation under aerobic conditions [4, 5]. The initial step in the aerobic catabolism of biphenyl by most microorganisms occurs via oxidation of biphenyl at 2, 3-position to a *cis*-dihydrodiol, followed by dehydrogenation to 2,3-dihydroxybiphenyl. The ring is cleaved to form 2-hydroxy- 6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA, the ring *meta*-cleavage product, a yellow intermediate), which is then hydrolyzed to benzoic acid and 2-hydroxypenta-2,4- dienoate [6,7].

The genes encoding the enzymes for the biphenyl upper pathway (termed *bph*) were first cloned from *Pseudomonas pseudoalcaligenes* KF707 and later from *Burkholderia* sp. LB400 , which is now reclassified as *Burkholderia xenovorans* strain LB400. To date, four types of *bph* gene clusters have been cloned [6]. But the research works on biphenyl/PCBs degradation and *bph* genes have been mainly limited to the genus *Pseudomonas*, *Burkholderia*, and *Rhodococcus*. Therefore, it is necessary to isolate novel microorganisms and clone *bph* gene to achieve effective bioremediation and biosensing of the biphenyl/PCBs pollution [9, 10].

Enzymes are the most widely used biological sensing element in the fabrication of biosensor. Although purified enzymes have very high specificity for their substrate or inhibitors, their application in biosensor construction may be limited by tedious, time consuming and coasty enzyme purification, requirement of multiple enzymes to generate the measurable product or need of cofactor/Coenzyme Microorganisms provide an ideal alternative to these bottle necks such as microbial biosensors[11, 12].

Bacteria can be used as biosensors to demonstrate the toxicity of a variety of environmental media including soil, sediment, and water by coupling bacteria to transducers that convert a cellular response into detectable signals [13]. These bacterial biosensors are engineered by pairing a reporter gene that generates a signal with a contaminant sensing component [14, 15].

When the biosensor is exposed to such a sensing component, it stimulates the reporter gene through a biochemical pathway in the cell. The reporter gene then produces a measurable response, such as emitting visible light, which is indicative of the degree of contaminant [16]. In the present work, such biosensor was prepared by transformation of plasmid from recombinant *E.coli DH5α* (*E.coli DH5α* transformed using pUC18 cloned with GFP gene) to the *S.maltophilia* which possesses a good biphenyl degradative activity. This transformed strain may effectively be used as a promising tool for biosensing biphenyl, scanty of such type of work has been reported in the literature.

## MATERIALS AND METHODS

### Isolation and identification

For isolation of biphenyl degrading bacteria, minimal broth containing salts supporting the growth of the organism was used. Biphenyl was added as a sole source of carbon in the medium [17]. As biphenyl is insoluble in water it was first dissolved in diethyl ether and this mixture was added in the minimal medium. Activated sludge sample was added in the medium for the isolation of organisms. The most efficient organism was identified as *Stenotrophomonas maltophilia* using VITEK 2 SYSTEMS VERSION 05.02.

### Biodegradation of biphenyl

Under aseptic conditions 1000ml sterile minimal medium was equally distributed in ten sterile flasks.

Biphenyl was added at a concentration of 50 ppm in each flask from stock solution of biphenyl, then 24 hrs old culture of organisms was inoculated in the flasks and incubated at 27$^{o}$C for 5 to 6 days.

**Estimation of biphenyl**

The reduction in biphenyl concentration was estimated using UV spectrophotometer in the range of 200 nm to 400nm every 24 hrs, utilizing one flask every time.

**Isolation of plasmid from *E. coli* DH5α**

**Materials:** 24-hour-old culture of GFP cloned *E coli DH5α* (Banglore Genei), sterile Luria Bertani (LB) ampicillin agar plates. Reagents used were alkaline lysis solution I (Glucose, Tris EDTA Buffer : pH 8.0) [50 mM Glucose; Glucose-18 mg, Sterile distilled water-100ml], 25mM Tris HCl [ Tris HCl- 302.85 mg, sterile distilled water-100ml], 10mM EDTA; [EDTA-372.24 mg, sterile distilled water-100ml]. Alkaline lysis solution II (SDS- NaOH) [100mg-SDS in 10 ml of 0.2N-NaOH], Alkaline lysis solution III (Potassium Acetate Solution) [5M Potassium Acetate- 60 ml, Glacial Acetic Acid-11.5ml, Sterile distilled water-28.5 ml], Alkaline lysis solution IV (Tris EDTA buffer) [10mM Tris; Tris-121.14 mg, Sterile distilled water-100ml], 1mM EDTA; [EDTA-37.224mg, Sterile distilled water-100ml] and Isopropanol.

**Protocol:** The recombinant *E coli DH5α* (*E coli DH5α* transformed using pUC18 cloned with GFPgene from jelly fish, as vector) was streaked on Luria Bertani (LB) Ampicillin agar plate and incubated at 37$^{o}$C for 48 hours. The plates were kept for 4 to 5 days to obtain good cell mass. Dense suspension of the organism was prepared in approx.0.3ml of Glucose Tris EDTA Buffer. 0.6 ml of Lysis buffer was added and incubated on ice bath for 5 minutes. 0.4ml of potassium acetate solution was added and kept on ice for 10 minutes. The tubes were centrifuged at 8000 rpm for 5 minutes at 4$^{o}$C. The supernatant was transferred to fresh tubes. This fractionation step separated the plasmid DNA from the cellular debris and chromosomal DNA in the pellet. 0.5 ml of isopropanol was then added. The tubes were incubated at room temperature for 2 minutes and centrifuged at 8000 rpm for 5 minutes at 4$^{o}$C. Then 1ml of ice cold Ethanol was added. The tubes were mixed by inverting several times and centrifuged for 1 min at 8000 rpm. The supernatant was discarded. Appendorf tube was air dried for 5 minutes. Then 0.3-0.5 ml of Tris EDTA buffer was added. The plasmid sample stored at -20$^{o}$C was ready to use.

**Detection of plasmid DNA by Agarose Gel Electrophoresis**

1% Agarose gel was used for the electrophoresis. Plasmid was separated and showed a single orange coloured band indicating the integrity and that plasmid was not degraded during the preparation used for plasmid isolation and this isolated gfp plasmid was used for transformation.

**Competence induction in S. maltophilia**

Small amount of growth of *S.maltophilia* was inoculated from slant into 500ml flask containing 100ml sterile nutrient broth. Broth was incubated on shaker incubator at 200 rpm for 6-8 hrs at 27°C.Then the cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C and resuspended in chilled 20 ml (1:1) mixture of 150mM $CaCl_2$ and 100 mM $MgCl_2$ solution, and kept at 4°C for 10min.Cells were then harvested by centrifugation and resuspended in 1ml (1:1) mixture of 150 mM $CaCl_2$ and 100mM $MgCl_2$ solutions and stored at 4$^{0}$C until further use [18].

**Transformation of plasmid in S.maltophilia**

1ml gfp plasmid preparation in TE buffer was added into 200µl of $CaCl_2$ treated cell preparation. The preparation was incubated at 4°C for 60min. and subjected to heat shock at 42°C for 2min in water bath then again incubated on ice bath for 1hr. The bacteria were then inoculated in 1ml of sterile SOC medium(SOB, a

version of LB with added 20 mM glucose) and incubated at 27°C for 1hr, this allowed the cells to recover and express the plasmid proteins. These bacteria were plated out on Cetrimide agar plate for scoring the total number of cells surviving the treatment. Transformant *S.maltophilia* were then selected on Cetrimide agar and subsequently confirmed by their green fluorescence under UV light [18,19]

Bac-test laboratory

bioMerieux Customer:
System #:

**Laboratory Report**

Printed Mar 24, 2012 06:58 IST
Printed by: bactest

Isolate Group: IS/1203/113-1

Patient ID: IS/1203/113
Bench: AIR SAMPLE

Bionumber: 1022103001540020
Selected Organism: Stenotrophomonas maltophilia

| Comments: | |
|---|---|

| Identification Information | Card: GN | Lot Number: 241215210 | Expires: Nov 4, 2012 12:00 IST |
|---|---|---|---|
| | Completed: Mar 23, 2012 17:46 IST | Status: Final | Analysis Time: 5.00 hours |
| Selected Organism | 99% Probability | Stenotrophomonas maltophilia | |
| | Bionumber: 1022103001540020 | | Confidence: Excellent identification |
| SRF Organism | | | |
| Analysis Organisms and Tests to Separate: | | | |
| Analysis Messages: | | | |
| Contraindicating Typical Biopattern(s) | | | |

Biochemical Details

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | APPA | + | 3 | ADO | - | 4 | PyrA | - | 5 | lARL | - | 7 | dCEL | - | 9 | BGAL | - |
| 10 | H2S | - | 11 | BNAG | + | 12 | AGLTp | - | 13 | dGLU | - | 14 | GGT | + | 15 | OFF | - |
| 17 | BGLU | + | 18 | dMAL | - | 19 | dMAN | - | 20 | dMNE | - | 21 | BXYL | - | 22 | BAlap | - |
| 23 | ProA | + | 26 | LIP | + | 27 | PLE | - | 29 | TyrA | - | 31 | URE | - | 32 | dSOR | - |
| 33 | SAC | - | 34 | dTAG | - | 35 | dTRE | - | 36 | CIT | + | 37 | MNT | - | 39 | 5KG | - |
| 40 | ILATk | + | 41 | AGLU | - | 42 | SUCT | + | 43 | NAGA | - | 44 | AGAL | - | 45 | PHOS | + |
| 46 | GlyA | - | 47 | ODC | - | 48 | LDC | - | 53 | IHISa | - | 56 | CMT | - | 57 | BGUR | - |
| 58 | O129R | - | 59 | GGAA | + | 61 | IMLTa | - | 62 | ELLM | - | 64 | ILATa | - | | | |

Installed VITEK 2 Systems Version: 05.02
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 1

**Table 1** VITEK 2 SYSTEMS analysis report for *Stenotrophomonas maltophilia.*

## RESULTS AND DISCUSSION

### Isolation and identification

The biphenyl degrading organism was identified as *Stenotrophomonas maltophilia* using VITEK 2 SYSTEMS version 05.02 (Table-1). There are many bacterial strains from the genera *Psedomonas, Vibrio, Aeromonas, Micrococcus, Acinetobacter, Bacillus, Rhodococcus, Mycobacterium* and *Sreptomyces* that degrade biphenyl or mono-,di-,tri- chlorinated PCBs. [08,20] but no reports regarding *S.maltophilia* degrading biphenyl were found.

### Degradation of biphenyl

The biphenyl degradation *by S. maltophilia wild* and the *transformed S. maltophilia* was estimated using UV spectrophotometer summerized in fig.1 and fig.2 by the overlay spectrum graph.

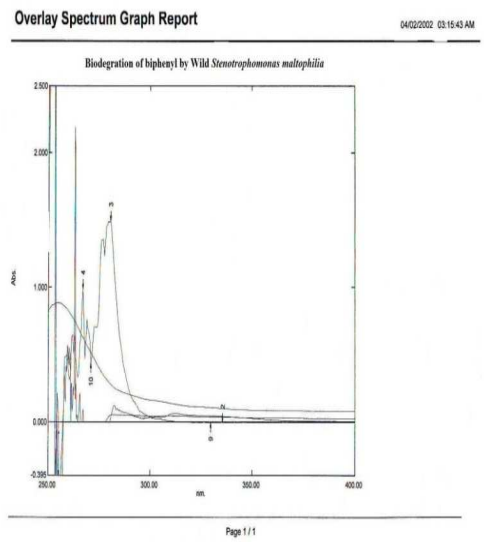


**Fig.1:** Degradation of biphenyl by wild *S. maltophilia.*

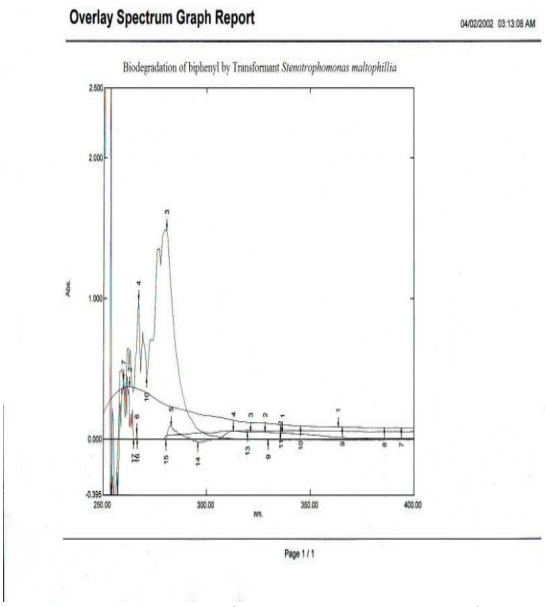


**Fig.2:** Degradation of the biphenyl by transformed *S. maltophilia.*

It was found that *S.maltophilia* wild could degrade 89.9% of biphenyl (Fig.1) while *E. Coli DH5α* degraded 24% biphenyl within four days.

**Fig.3** *S.maltophilia* culture (wild).

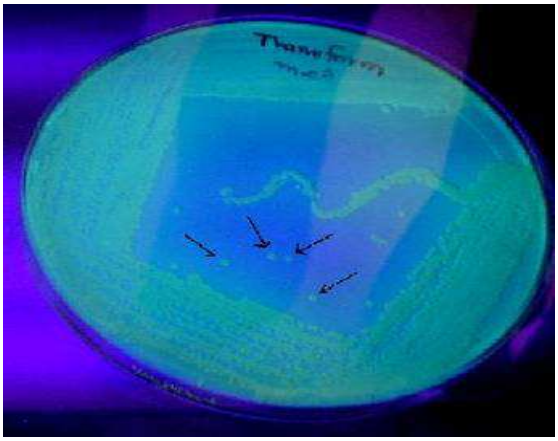

**Fig.4** *S. maltophilia* culture (transformed)

After transformation of *S. maltophilia* with gfp plasmid from *E. Coli DH5α* i.e. transformed *S. maltophilia* degraded 97.5% of the biphenyl (Fig.2) and also the organism exhibited fluorescence under UV light (Fig.3, 4)[21]. Microorganisms have been integrated with a variety of transducers such as ampherometric, potentiometric, calorimetric, conductiometric, luminescence and fluorescence to construct biosensor devices. Several review papers and text chapters addressing microbial biosensor development have been published [11,21,22,23] but there are no reports on green fluorescence pigment based *S. maltophilia* biosensor for biphenyl degradation.

## CONCLUSIONS AND DISCUSSION

Biphenyl degrading *S. maltophilia* was successfully isolated from the activated sludge sample. As per literature survey *S. maltophilia* has not so far been reported to degrade biphenyl. After transformation, *S.maltophilia* was able to give fluorescence as well as 97.5% of biphenyl degradation activity thus this capacity of the transformed organism can be used as an efficient tool for biodegradation, bioremediation as well as for biosensing biphenyl. Efficient biosensors for other chemical pollutants can also be prepared by adopting like methodology.

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