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EVIDENCE OF AEROBIC DEGRADATION OF POLYCHLORINATED BIPHENYL AS GROWTH SUBSTRATE BY NEW BACTERIA *STENOTROPHOMONAS* SP. JSG1 AND *CUPRIAVIDUS TAIWANENSIS* JSG2

Selvaraj GANESH-KUMAR, Robinson David JEBAKUMAR SOLOMON[1], Karuppanan KALIMUTHU & Jayaraj VIMALAN
[1]Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai-21, Tamilnadu, India. e-mail: jsolomon@mrna.tn.nic.in., ganesh_kumarbt@yahoo.co.in.

Abstract: New bacterial strains capable of degrading polychlorinated biphenyls (PCBs) were isolated from soil contaminated with waste from dye industry. The enriched colonies were exposed to 2,3-dihydroxybiphenyl (2,3-DHBP) and produced yellow metabolite of biphenyl catabolic pathway. Based on their morphological, chemotaxonomic characteristics and 16S rRNA phylogenetic typing, the strains were identified as *Stenotrophomonas* sp. JSG1 and *Cupriavidus taiwanensis* JSG2. They are able to utilize *ortho*- and *para*-substituted mono-, di-, and trichlorinated biphenyls (4-Chlorobiphenyl, 4,4'-Chlorobiphenyl and 2,4',5-Chlorobiphenyl respectively) as sole carbon and energy sources. The presence of 2,3-Dihydroxy biphenyl dioxygenase (2,3-DHBD) was confirmed on crude protein extract of PCB degraders and their molecular weight is about 36 kDa. GC analysis revealed that both organisms possess significant PCB degrading activity. The active metabolic rate of the strains were identified by the production of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) and their level estimated at a range of time points. Investigations into the genetic systems controlling the degradation of polychlorinated biphenyl on these organisms and their co-metabolic study are in progress.

Keywords: HOPDA, Polychlorinated biphenyl, 2, 3-Biphenyl dioxygenase, 2,3-DHBP

1. INTRODUCTION

Polychlorinated biphenyls (PCBs) are synthetic aromatic compounds exploited as coolants, insulating fluids for transformers, capacitors and used in flame retardant, waxes, resin, paints, copy paper, sealants for wood and for cement surfaces etc. On the other hand, PCBs are widespread environmental pollutants and cause some hazardous effects on landfill, soil and bottom sediments. PCBs are carcinogenic, persist in land and their half life reported from three months to 47 years. As a result of their consequence, they have created public and scientific concern for several decades. For the industrial usage, PCBs are started to produce on 1954 in UK and 1.1 million tons of PCBs produced in worldwide at 1984 (Alcock et al., 1993; Erickson 1997; EA 2007). They are very stable upto 350 °C and are resistant to acid, base and oxidative effects. Hence, PCBs are very difficult to complete degradation (Lauber et al., 1986). Conventional remediation methods such as land filling, incineration etc, are very expensive, inept and lead to secondary pollution (Mhiri et al., 1997; Robinson and Len, 1994). As a result of this, current research aims to progress the bioremediation.

Polychlorinated biphenyls are environmentally degraded by both oxic and anoxic (reductive dehalogenation) circumstances using various metabolic pathways (Strand 1990). PCBs are mainly used by microorganisms because of three possibilities i.e., aerobic microbes can use PCB as a substrate, co-metabolism at oxic condition and anaerobically by dehalogenation. At an aerobic condition, PCBs are degraded by both gram negative and gram positive bacteria using meta and ortho cleavage pathways (Sylvestre 1995; Sierra et al., 2003; Rein et al., 2007; Adebusoye et al., 2008).

Generally, biphenyl or PCB degradation is started with biphenyl 2,3-dioxygenase encoded by *bphA* to attack the 2,3 position of chlorinated biphenyl or biphenyl ring to cis-2,3-dihydro-2,3-dihydroxybiphenyl. Cis-dihydrodiol is then dehydrogenated to 2,3-dihydroxy biphenyl by dehydrogenase (*bphB*). The 2,3-dihydroxybiphenyl is then cleaved at the 1,2 position by 2,3-dihydroxybiphenyl dioxygenase (*bphC*) and yield yellow biphenyl-derived cleavage product known as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). This yellow product is hydrolyzed to yield benzoate and 2-hydroxypenta-2,4-dienaote by the gene *bphD* encoding hydrolase (Gibson et al., 1973; Pieper 2005). Higher chlorinated compounds undergoes into lower chlorinated compounds on oxic conditions.

This work was undertaken to characterize the new aerobic PCB degraders *Stenotrophomonas* sp. JSG1 and *Cupriavidus taiwanensis* JSG2 with the unique capability to utilize the *ortho*- and *para*-substituted mono-, di-, and trichlorinated biphenyls and to study the possibility of using this strains in mixed cultures for the degradation of a wide range of chlorinated biphenyls.

2. MATERIALS AND METHODS

2.1. Chemicals

Analytical standard PCB congeners (Oekanal grade) such as Biphenyl, 4-Chlorobiphenyl (4-CB), 4,4'-Chlorobiphenyl (4,4'-CB), 2,4',5-Chlorobiphenyl (2,4',5-CB) and purum grade 2,3-Dihydroxybiphenyl (98%, HPLC) were purchased from Sigma-Aldrich, Bangalore, India. HPLC grade solvents were used for PCB degradation and extraction methods.

2.2. Culture medium

Enrichments and degradation experiments were carried out in liquid Phosphate buffer mineral salt medium (PAS) according to Bedard et al. (1986). Finally, the required molten biphenyl crystals were added. Molten biphenyl crystals were added on the lid of the petri dish to prepare the PAS plates (1.8% agar).

2.3. Microcosm enrichment

Soil samples were collected from beneath the ground, which are historically contaminated (10-15 years) places of dye industry, Sivakasi, Tamil Nadu, Southern India. To obtain the enrichment culture, 2g of soil was mixed to 18 ml of PAS medium in 100ml Erlenmeyer flasks with 0.01% biphenyl and incubated at 30°C, 120 rpm. After 10 days, enrichment cultures (10%) were transferred to fresh PAS medium containing biphenyl (Petric et al., 2007). Subsequent transfers were made using 1% of inoculum and these steps repeated until the HOPDA produced on the medium. The biphenyl crystals were gradually increased upto 0.15%, while every transfer of mother culture.

2.4. Bacterial strains expressed *bphC* (2, 3-DHBD) activity

The isolated bacterial colonies on PAS agar plates were analyzed for the activity of 2,3-Dihydroxy biphenyl dioxygenase (2,3-DHBD), which is encoded by *bphC* gene involved in the biphenyl pathway. The bacterial colonies were tested by the addition of 2, 3-DHBP, an aqueous solution containing 1 mg/mL of 2, 3-DHBP and 0.1 ml/mL of acetone (Petric et al., 2007).

2.5. 16S rRNA gene sequencing and phylogenetic analysis

The total genomic DNA was extracted from scraped bacterial colonies using guanidium thiocyanate method (Pitcher et al., 1989). 16S rRNA genes in the harvested genomic DNA were amplified by the universal primers targeted to conserved regions of the 16S rRNA gene [forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and a reverse primer 1492R (5'-AAGGAGGTGATCCAGCCGCA-3')] using the BIORAD thermocycler. PCR amplified 16S rDNA were sequenced with the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster, CA) using an ABI 3100 DNA sequencer (Applied Biosystems). DNA sequences were analyzed by the NCBI BLAST database, Ribosomal Database Project (RDP), CLUSTALW and PHYLIP version 3.68. Bootstrap analysis was used to determine the confidence values of nodes of phylogenetic tree using 1000 resamplings (Felsenstein, 1985).

2.6. Nucleotide sequence accession number

The 16S rRNA gene sequences of *Stenotrophomonas* sp. JSG1 and *Cupriavidus taiwanensis* JSG2 were deposited at National Center for Biotechnology Information (NCBI) under the accession number of GU202014 and GU202015 respectively.

2.7. Resting cell incubation assay

Bacterial cells were grown for 40h incubation on PAS medium containing biphenyl (0.2%) and the culture was filtered via glass wool to separate the biphenyl crystals. Cells were harvested by centrifugation (15,000g, 15min, 4°C), then washed trice with 50 mM sodium phosphate buffer (pH 7.5). Washed cells were resuspended in the same buffer at an absorbance at 600nm of 2.0. Cell culture (5%) was inoculated on PAS medium containing 4-CB, 4,4'-CB and 2,4',5-CB (5 mg/L) on 15 mL screw cap bottles. Control cells were killed by autoclaving at 121°C for 15 min prior to preincubation or by adding perchloric acid (final concentration at 0.7%) to stop the reaction. Finally, both the control and sample cultures were incubated at 30°C, 120 rpm.

2.8. Measuring the HOPDA level and Growth rate

Suspensions of PCB strains incubated with 4-CB, 4,4'-CB and 2, 4',5-CB were filtered via glass wool, centrifuged and the aqueous supernatant was scanned by the UV-spectrophotometer (U1100-Hitachi) at a range of time intervals at 434 nm characteristic λ-max of the PCB-derived cleavage product known as HOPDA (Xiuqing et al., 2004). Suspensions of PCB degraders incubated with 4-CB, 4,4'-CB and 2,4',5-CB on PAS mineral medium were scanned to observe the growth rate after filtering via glass wool at 600 nm at various time intervals. Killed cells were taken as control.

2.9. PCB extraction and GC analysis

After the incubation with 4,4'-CB (triplicated), cell suspension was added with perchloric acid (final concentration at 0.7%). PCB extractions were performed by addition of 0.1 ml of culture to a 15-ml Teflon coated screw cap tube containing 6 ml H_2O, 1 g sodium sulphate and 2.5 ml diethyl ether. The tubes were shaken for 1 h on a reciprocating shaker and centrifuged for 15 min at 700 × *g*. From the supernatant, 0.5 ml ether was transferred to a 5-ml screw cap tube containing 2 ml hexane and 0.5 g silica gel. The extracts were shaken for 30 min, centrifuged at 190 × *g* for 5 min, and transferred to GC vials (Lajoie et al., 1997). Sample extracts were compared with the control using a gas chromatograph (GC-7610, Chemito, India) equipped with a flame ionization detector (FID) and injector, both of which were kept at 300°C. A HT-8 capillary column (50 m×0.22 mm i.d.) was used with nitrogen as the carrier gas (2 mL/min) and as the make-up gas (28 ml/min). Injection of 1µL of the sample (by 10µL Hamilton syringe) was performed using the split less mode. The column oven temperature was kept at 50°C for 2 min, then raised to 80°C at a rate of 10°C/min and then to 225°C at a rate of 6°C/min, and then kept at 225°C for 35 min.

2.10. Checking the presence of 2, 3-dioxygenase enzyme

Cell suspension in sodium phosphate buffer was prepared using above described method as in Resting cell incubation assay. Cells were disrupted by ultra sonication in ice pack and the cell debris deposited by centrifugation at 13835 × *g* for 30 min at 4°C. The collected crude protein extract in supernatant was stored at -20°C (typically 4-8 h). Total protein concentration was estimated using the Bradford method by taking BSA as standard. The enzyme activity was assayed in the mixture comprised of 100 µl crude extract, 90 µl 50 mM Tris-Cl buffer with 10% acetone and 10 µL of substrate (440 µM 2,3-DHBP) at 37°C (Totevova et al., 2002). 2,3-dioxygenase activity was examined at 434 nm by UV-VIS spectrophotometer (Khan & Walia, 1989).

2.11. SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with Mini Slab System (Biotech R&D Laboratories, Yercaud, India) and 12% polyacrylamide gel was used as the uniform separating gel (0.75 mm thickness) and overlaid with 5% stacking gel. The subunit molecular mass was determined by SDS-PAGE after treating the crude protein with 5% SDS and then heating at 100°C for 3-4 min. Around 20 µg of crude extract protein was loaded in each well. Protein bands in the gel were stained and visualized with Comassie brilliant R-250.

3. RESULTS AND DISCUSSION

3.1. Enrichment and selection of PCB degraders

The bacterial strains utilized in this study were isolated by using biphenyl as the sole source of energy. During the microcosm enrichment (4-7 sub culturing, triplicated), periodically monitored the production of meta cleavage yellow product, HOPDA. Initially, yellow colour was produced with in 3 days from the onset of incubation, and then the time shortened to 20 h. After the microcosm enrichment, the mixed cultures were purely separated by serial dilution method. The soil sample isolated from industrial dye polluted site given fastest growing strain of JSG1 and JSG2. The experiment culture medium had the two phases- yellow stage and white stage, in which strains started to produce meta cleavage yellow product during yellow stage and then the organisms vanished their colour completely and posses mature bio cells during white stage.

3.2. Characterization of selected bacterial communities

After the incubation of PCB degraders on PAS agar plates with molten biphenyl supplied on the lid of Petri dishes, the ability to express their *bphC* gene was observed. This gene encoding the 2,3-dihydroxybiphenyl dioxygenase enzyme is able to produce yellow meta cleavage product by reacting with the substrate 2,3-dihydroxybiphenyl. Hence, the substrate was sprayed on the bacterial colonies and the positive colonies were turned into yellow colour within 1-3 min. This experiment confirmed the expression of *bphC* gene of bacterial colonies. The length of 16S rRNA gene sequence of JSG1 was 1392 bp and JSG2 was 1372 bp. The two enriched microorganisms shared its close resemblance to *Stenotrophomonas maltophilia* (AB008509) and *Cupriavidus taiwanensis* JC4 (AM905275) in phylogenetic tree by Neighbour-joining method (Figure 1). The level of sequence similarity among the related species ranged from 97.4% to 99.8% and they were identified as *Stenotrophomonas* sp. JSG1 and *Cupriavidus taiwanensis* JSG2 (Table 1). Their biochemical characters were also studied (Table 2).

3.3. Spectral analysis

During 24h of incubation with 4-CB, 4,4'-CB and 2,4',5-CB on mineral medium, the level of growth rate and release of meta cleavage yellow product level was analyzed by taking the absorption values at various time intervals. The JSG1 strain had better result at both growth rate and meta cleavage yellow product release than the strain JSG2 (figure 2). The release of meta cleavage yellow product exhibited absorption at 389-435 nm, characteristic of the yellow Meta cleavage product.

As shown in figure 2, after 15 min of incubation with 4-CB, 4,4'-CB, JSG1 strain produced yellow colour metabolite. But the strain JSG2 produced the yellow metabolite after 45 min of incubation with 4-CB, 4,4'-CB (Figure 2 A, B). Both the bacterial strains started to produce yellow metabolite after 5 h of incubation with 2,4',5-CB. The growth rate of strain JSG1 with 4-CB, 4,4'-CB is faster than the 2,4',5-CB (Figure 2 C, D). On the whole, 4-CB is utilized faster than the other two PCB congeners. Biomass of the both bacterial strains stopped at one point has known as logarithmic phase. In this stage, bacteria had more biomass and the yellow color vanished completely. It is clear that during the degradation study chlorine ring attached to the PCB congeners influence the HOPDA and growth rate. Until recently, it was generally believed that the occurrence of more than one chlorine atoms prevented chlorinated biphenyls from serving as a sole source of carbon and energy for aerobic bacteria. But this study clearly states that, isolated new strains are capable of utilizing ortho- and *para*-substituted mono-, di-, and trichlorinated biphenyls as carbon sources.

Table 1 Biochemical characters of isolated PCB degraders

Biochemical characters	*Stenotrophomonas* sp. JSG1	*Cupriavidus taiwanensis* JSG2
Oxygen Requirement	aerobic	aerobic
Gram's test	gram-negative	gram-negative
Shape	rod-shaped	rod-shaped
Motility test	motile	motile
Catalase	+	+
Oxidase	+	+
Nitrate reduction	+	+
Gelatin	+	-
Starch	-	-
Urease	-	-
Indole	-	-
H_2S	-	-
Hemolytic test	-	-

* - negative, + positive

Table 2 Genetic similarity of PCB-degrading bacterial isolates derived from cloned sequences of 16S rDNA fragment of genomic DNA

Bacterial Strains	Tentative identity	Closest relationship	Bacterial subdivision	Similarity (%)	Genbank Accession number	Length (bp)
JSG1	*Stenotrophomonas* sp.	*Stenotrophomonas maltophilia*	γ-proteobacteria	97.4	GU202014	1392
JSG2	*Cupriavidus taiwanensis*	*Cupriavidus taiwanensis* JC4	β-proteobacteria	99.8	GU202015	1372

* bp - base pair

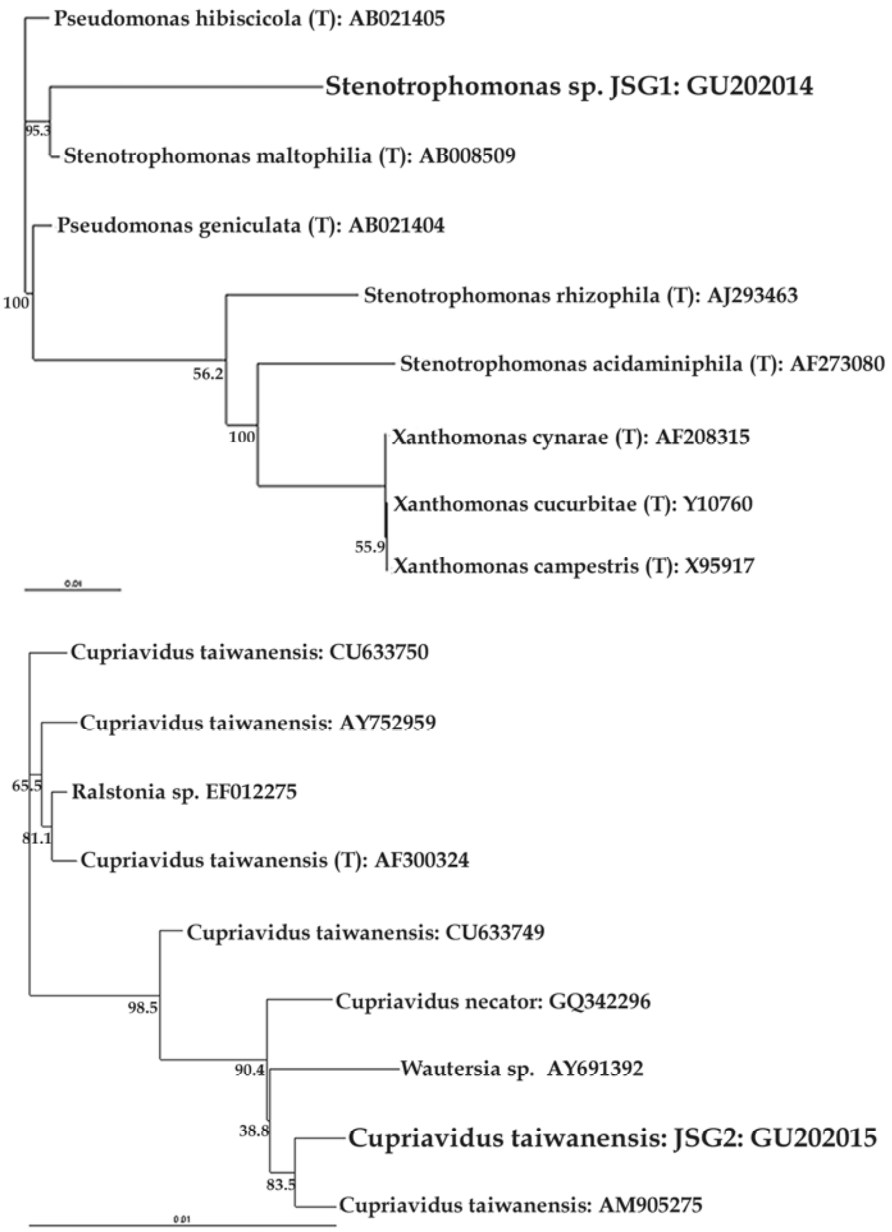

Figure 1. A Neighbor-Joining phylogenetic tree illustrating the clustering of 16S rRNA gene sequences of linage of *Stenotrophomonas* sp. JSG1 and *Cupriavidus taiwanensis* JSG2. The tree topology was analyzed from the distance matrix generated by Juker Canter method with bootstrapping of 1000 replicates using the PHYLIP package. Bootstrap values are depicted adjacent to each node and 0.1 scale bar indicates the nucleotide substitution level.

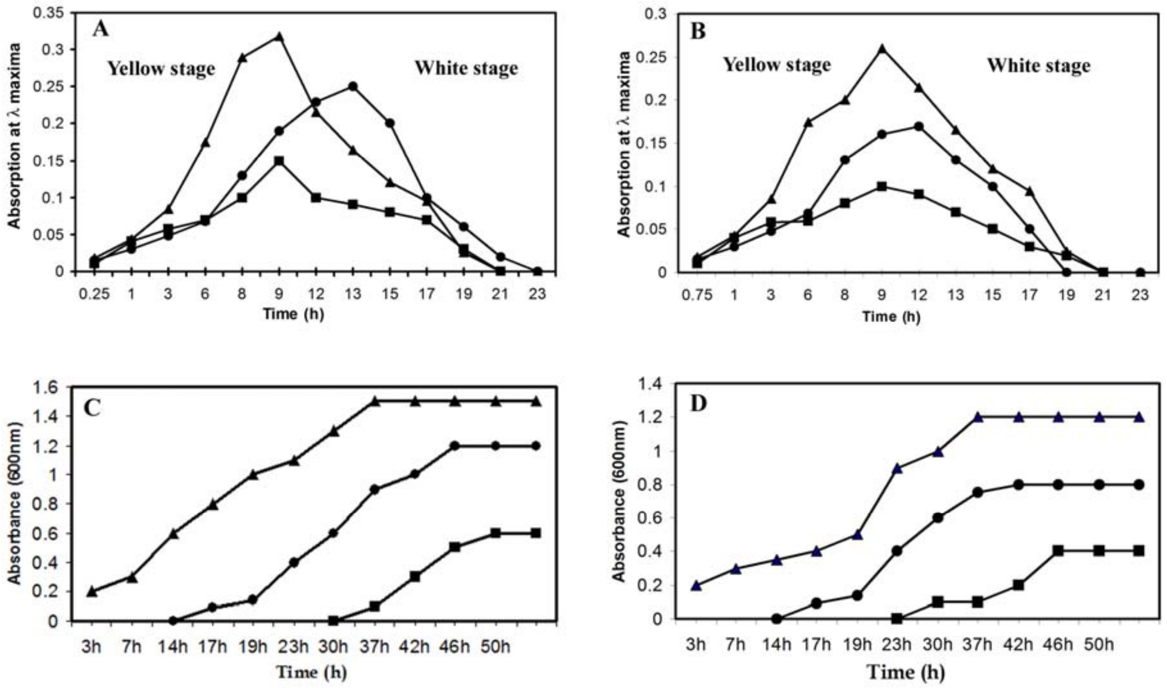

Figure 2. Diagram showing the spectrum of strain JSG1 (A) and strain JSG2 (B) on mineral medium containing 4-CB, 4,4'-CB, 2,4',5-CB released chlorinated HOPDA at various time intervals and growth rate of JSG1 (C) and JSG2 (D) on mineral medium containing 4-CB, 4,4'-CB, 2,4',5-CB at various time intervals (4-CB: 4-chlorobiphenyl (▲), 4,4'-CB: 4,4'-chlorobipheny l(●) and 2,4',5-CB: 2,4',5-chlorobiphenyl (■)).

3.4. Degradation of di-CB

PCB depletion study was determined in triplicated resting cell assays. 1µL of hexane extract incubated with di-CB was analyzed using Gas chromatography equipped with FID. JSG1 utilized 97% of di-CB within 5 days of incubation and JSG2 utilized 94% of di-CB with in 5 days of incubation as shown in Table 3. It is very clear that the isolated new strains JSG1 and JSG2 are able to degrade PCB congener effectively than the other organisms reported previously (Matthew et al., 2008). To the best of our knowledge this is the first report about growth pattern, HOPDA level and degradation of di-CB by these new organisms JSG1 and JSG2.

Table 3 Degradation of 4,4'-chlorobiphenyl by strains JSG1 and JSG2

Duration (day)	*Percent Degradation Remaining	
	strain JSG1	strain JSG2
1	100	100
2	37.4 ± 2.5	57.5±0.2
3	23.5 ± 1.2	26.7±0.8
4	10.2 ± 0.5	14.8±0.6
5	3.3 ± 1.4	5.8±0.8

**Note:* Percent degradation was calculated relative to the control (Heat Killed Cells). Values shown are mean ± SD for three replicates.

3.5. Enzyme assay

The crude proteins (1 mg) of two bacterial strains were checked for the presence of 2,3-biphenyl dioxygenase enzyme by the addition of 2,3-dihydroxy biphenyl (440 µM). After the addition of 2,3-dihydroxy biphenyl into the reaction mixture, yellow meta cleavage product was produced on JSG1 suspension within 3-4 min of incubation at 37°C and JSG2 strain produced yellow meta cleavage product within 7-9 min of incubation.

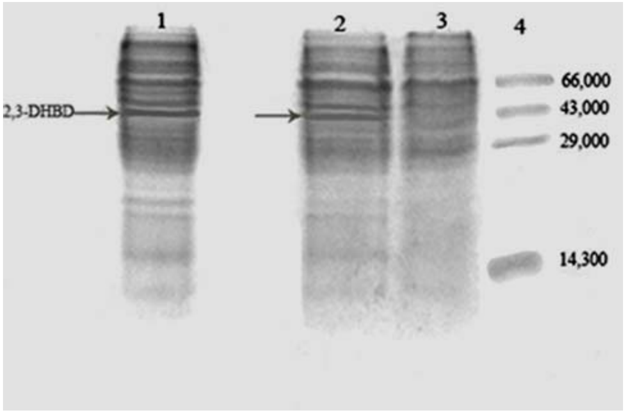

Figure 3. SDS-PAGE of 2, 3-DHBD crude extracts obtained from PCB degraders. Protein bands were stained with coomassie brilliant blue R-250. Lane 1: *Stenotrophomonas* sp. JSG1, Lane 2: *Cupriavidus taiwanensis* JSG2, Lane 3: control bacteria, Lane 4: MW ladder. The sizes of the peptides are given in kilo Dalton.

The optimum pH for the 2,3-DHBD enzyme reaction was 6.8 for both organisms. After the detection of dioxygenase enzyme, the crude extracts (20 µL) were resolved on 12% of SDS-PAGE gel on mini slab system. Resolved gel was stained with Comassie brilliant R-250. Previous studies proved that molecular weight of the 2,3-DHBD on SDS-PAGE are about 36 kDa (MeiYing et al., 2007). New JSG1 and JSG2 strains showed specific band (nearly 36 kDa) on the stained gel (Figure 3).

4. CONCLUSION

To conclude this study, we isolated and characterized the new strains *Stenotrophomonas* sp. JSG1 and *Cupriavidus taiwanensis* JSG2 from dye industry and they possess capacity to cleave chlorinated biphenyl by using the PCBs as a sole source of carbon, which is ever reported previously on these strains.

JSG1 had the high growth rate, fast lag phase and release of meta cleavage product in short time. Earlier reports state that *Rhodococcus* sp. started to produce yellow meta cleavage product (HOPDA) after 30 min, but strain JSG1 started to produce the HOPDA after 15 min and proved that this strain can able to degrade the di-CB faster (less than 5 days) than the earlier reports of other organisms (Matthew et al., 2008; Xiuqing et al., 2004). Confirmed by SDS-PAGE and enzyme assay, these aerobic strains have 2,3-DHBD enzyme and both the strains degrade polychlorinated biphenyl within short duration. All this shows that strain JSG1 and JSG2 are very efficient PCB degraders and can be used for compiling mixed cultures, significant to bioremediation of PCB contaminated soils. These new strains *Stenotrophomonas* sp. JSG1 and *Cupriavidus taiwanensis* JSG2, which possess unique enzymatic activities providing for the healthy utilization of 4, 4'-dichlorobiphenyl. Currently, we are focusing on the genetic systems controlling the degradation of polychlorinated biphenyl by these strains and their co-metabolic study is in progress in our laboratory.

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