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Optimization of 2,4 dichlorobiphenyl (PCB Congener) degradation by *Pseudomonas spp.*

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Abstract

Pseudomonas spp consortium (GSa and GSb) was optimised to degrade 2,4 dichlorbiphenyl (2,4 CB) in shake flask culture. The optimisation parameters such as concentration of the substrates, rotations per minute, effect of substrate, concentration of sodium glutamate, pH, temperature, for degradation of 2,4 dichlorobiphenyl were evaluated. The maximum degradation of 2, 4 CB was observed under optimised conditions such as 35°C, pH 7, rpm- 130, and the concentration of 2,4 CB was 90 ppm. Supplimentation of 0.2% sodium glutamate and Surfactant Tween 80 resulted in highest amount of 2,4 CB degradation which was measured spectrophotometrically. The amount of chloride release with increase in biomass was estimated by Mohr's argentometric method. Under these optimised conditions the consortium GSa and GSb had the potential to degrade 2, 4 CB, a PCB congener, perhaps a candidate for in-situ bioremediation.

Keywords: *Biodegradation; optimisation; 2,4 dichlorobiphenyl (2,4 CB); Pseudomonas spp. GSa and GSb*

1 Introduction

Polychlorinated biphenyls (PCB) have been known as persistent and widespread contaminant; represent one of the most serious environmental pollutant (Petric et al. 2007). PCB were produced in large quantities and used typically as mixtures in diverse substances such as plastics, carbon paper, coding systems, hydraulic fluids, transformers and fire retardants (Boyle et al., 1992). Their wide spread use resulted in heterogenecity, persistence, toxicity, carcinogenic potential, and tendency to bioaccumulate as they ascend the food chain, making them as major serious environmental pollutants (Abramowicz et al., 1990). Such pollution brings out serious and disastrous changes in composition of flora leading to lose their habitat (Baruah and Sarma, 1996). Toxic contaminant present in soil in high concentration reduces the degradation of PCB by indigenous bacteria and hence the population and efficiency are affected. To overcome this problem, reintroduction after enrichment of indigenous microorganisms isolated from a contaminant site is necessary (Mishra et al., 2001; Somaraja et al., 2013). It is unlikely, that identical xenobiotic degrading bacteria are distributed around the globe, although differing ambient environmental conditions, soil composition, organic carbon soil inputs and many other factors. In view of the wider geochemical and physical variations, tropical microrganisms may exhibit a fascinating metabolic diversity and possess novel catabolic properties for real time degradation of PCB. Therefore, in present study a consortium of Pseudomonas spp isolated from transformer polluted sites, was grown exponentially on 2,4 dichlorobiphenyl as a sole carbon and energy sources under aerobic condition. The coexisting GSa and GSb bacteria was able to transform 2,4 CB extensively and therefore physical and nutritional conditions were optimised for maximum degradation of 2, 4 Chlorobiphenyl.

2 Material and methods

Two isolates of *Pseudomonas spp* (GSa and GSb) which was previously identified by rDNA typing, maintained in our laboratory was optimized for 2,4 CB degradation

2.1 Growth curve

Growth curves of the consortium GSa and GSb was determined in Seubert mineral medium (SMS) (Seubert, 1960) and enumerated the bacteria at regular intervals (every hour) for 48 hr spectrophotometrically (600 nm). When the logarithms of the viable cells are plotted against time, a growth curve was obtained, subsequently at each interval the release of chloride ion was estimated (Figure 1).

2.2 Inoculum preparation for optimization of process parameters

About 100 l (0.1 ml) of growth medium from log phase (after 36 hr) was inoculated to the Seubert mineral medium, different parameters were optimised for effective and maximum degradation such as carbon source, co-substrate, different pH, temperature, substrates and incubation at different rpm and the enzyme assay. For biodegradation study the medium was supplemented with 2,4 dichlorobiphenyl (100 ppm).

2.3 Optimisation parameters

Surfactant: To examine the effect of surfactant, Tween-80 was used in 2, 4 CB degradation study. Rpm: To find out the optimum rpm for efficient degradation 2,4 CB (100 ppm) degradation GSa and GSb consortium was inoculated to SMS medium and was adjusted to 100, 120, 130 rpm. 2,4 CB: The effect of 2,4 CB on bacterial growth was evaluated using 100 ml of SMS medium

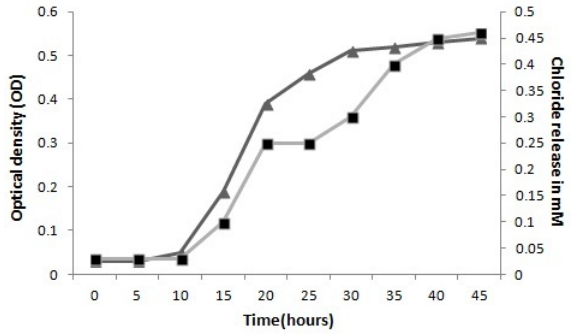

Figure 1a: Growth Curve of the Consortium (GSa and GSb) (▲) and Chloride release (■) with 2, 4 CB as substrate on SMS medium with tween 80

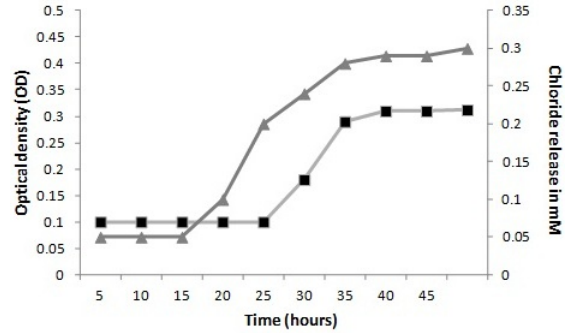

Figure 1b: Growth Curve of the Consortium (GSa and GSb) (▲) and Chloride release (■) with 2, 4 CB as substrate on SMS medium without tween 80

containing flasks supplemented with different concentration of 2,4 CB (30 ppm to 130 ppm). After 40 hr of incubation, the bacterial growth was measured (600 nm) spectrophotometrically and the chloride release was determined by Mohr's argentometric method (Marcin and Stanislaw, 1999; Greenberg et al., 1992). Additional carbon source: Different additional carbon sources like glucose, maltose, mannitol and sodium glutamate was used (0.5%) to study their effects on degradation of 2,4 CB. SMS medium was incorporated different carbon sources containing 2,4 CB (100 ppm) and were inoculated with the consortium of GSa and GSb and incubated/40 hr. Different concentrations of carbon; In order to optimize the carbon source for maximum degradation, the medium was supplemented with different concentration of carbon source to 2,4 CB (100 ppm) and the optimum concentration was estimated spectrophotometrically. pH: For maximum 2,4 CB degradation, the pH of the SMS medium with 2,4 CB (100 ppm) was adjusted to 5, 6, 7, 8 pH and the consortium was inoculated. The optimum pH for maximum degradation was measured spectrophotometrically. Temperature; For maximum degradation of 2,4 CB, the temperature of the SMS medium with 2,4 CB (100 ppm) was adjusted to 25°C, 28°C, 35°C, 45°C and the consortium was inoculated. Growth of the consortium degrading 2,4 CB on different substrates: Substrate specificity of the consortium (GSa and GSb) was determined in SMS medium with 4 mM of 2,4 CBA, 2 CBA, 4 CBA, toluene, benzene, biphenyl (0.1g/L) and the consortium was inoculated.

2.4 Determination of Hydroxyl-6-oxo-6 penta 2, 4 dienoic acid (HOPDA)

Suspensions of the consortium was incubated with 2,4 CB were filtered through glass wool, centrifuged and the aqueous supernatant was analysed spectrophotometrically (350-450 nm). SMS medium without 2,4 CB was used as a control. Preparation of cell free extract: The consortium (GSa and GSb) was harvested separately at its log phase of growth and centrifuged 5000 rpm/15 min). The cells were washed repeatedly with 50 mM sodium phosphate buffer, (pH 7.2) and suspended in 0.1 M KH_2PO_4 buffer, (pH 7.2) containing 1 mM ascorbic acid, 10% acetone, 10% glycerol and 100 M ferrous sulphate and later, the cells were lysed by sonication (Vibracell, sonics and materials, CT, USA, model VC 130) (4min/4°C). The cell debris and unbroken cells were separated by centrifugation (10,000 rpm/20 min). The resulting supernatant was used as crude source of enzymes. The protein concentration in this supernatant was estimated according to Bradford method.

2.5 Enzyme assay

2,4 dichlorobiphenyloxidoredcutase activity was measured after 25 hr and 35 hr of incubation in SMS. The supernatant of the culture media was used as crude enzyme source. The reaction mixture (4 ml) contained 25 mM phosphate buffer (pH 7.2), 0.17 mM NADPH, 0.047 mg of enzyme, and the reaction was initiated by adding 0.04 mM of 2,4 CB. Enzyme activity was measured with decrease in absorbance at 340 nm due to substrate dependent oxidation of NADPH (35°C). One enzyme unit was defined as the amount of enzyme which in presence of 2,4, CB causing oxidation of 1mol NADPH/minute. The oxidation of NADPH in the presence of phenol (Hydroxylase) was monitored after 25 hr and 35 hr (340 nm). The reaction mixture (4 ml) contained 50 mM KH_2PO_4 buffer (pH 7.2), 0.17 mM NADPH and 0.1 M phenol, equilibrated (35°C) before addition of the cell extract (0.04mg) (Gibson et al., 1990). 2,4 dichlorobiphenyl dehalogenase activity was measured in a reaction mixture (5ml) containing 25 mM phosphate buffer (pH 7.2), 0.4 mM 2,4 CB (substrate) and enzyme extract (0.04mg). The reaction mixture was equilibrated at 35°C for 10 min and the enzyme extract was added. Further, free halide was estimated according to Mohr's argentometric method (Marcin and Stanislaw, 1999).

3 Results

To optimize the 2,4 CB degradation by the consortium GSa and GSb, various parameters were evaluated such as, effect of surfactant, different concentration of the substrates, rotation per minute, effect of different substrates, concentration of sodium glutamate, pH and temperature.

3.1 Optimisation parameters

Surfactant: The assimilation of 2,4 dichlorbiphenyl was increased using surfactant Tween 80 (0.01 ml) which was evident with 0.45 mM chloride release (Fig.

1). Further, the maximum biomass of the consortium was obtained at 130 rpm compared to 100 and 110 rpm. Effect of different concentration of 2,4 CB: In order to determine the tolerance levels of the consortium, different initial concentration of 2,4 CB was evaluated from 30 to 130 ppm. The consortium was able to tolerate 130 ppm of 2,4 CB (Fig. 2). However, a maximum growth rate was observed at 90 ppm.

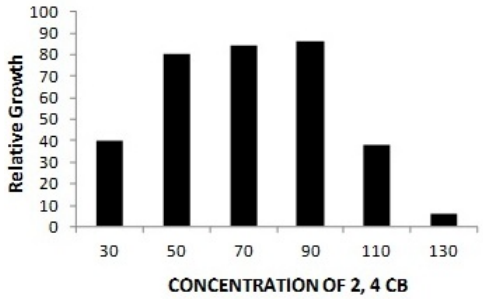

Figure 2: *Pseudomonas spp.* (GSa and GSb) growth on different concentration of 2, 4 dichlorobiphenyl

Further, four different carbon source viz., glucose, maltose, mannitol and sodium glutamate were used to study the effect on 2,4 CB degradation. There was a slight increase in the 2,4 CB degradation by supplementing sodium glutamate as an additional carbon source, which was detected by increase in OD (600 nm) and release of chloride (Table 1). And, optimum concentration of sodium glutamate for maximum degradation by the consortium was examined (0.1 to 0.4 percent) (Table 2). At 0.2% concentration an optimum growth with maximum chloride release of 0.49 mM was observed (0.48 OD). Further, the effect of pH on 2,4 CB biodegradation by the consortium was assessed. Highest cell density (0.53 OD), and the highest chloride release (0.45 mM) were obtained at pH 7, indicating pH preference of the consortium (Fig. 3 and 4; Table 3 and 4).

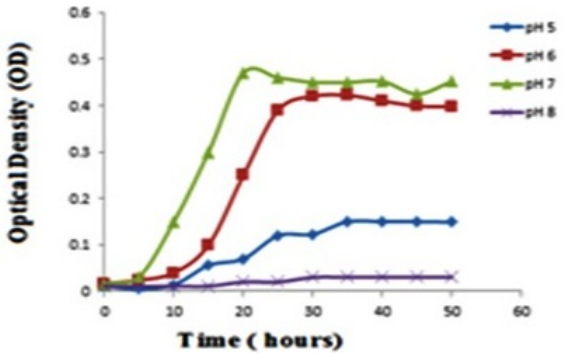

Figure 3:Optimization of growth of the consortium at different pH

Temperature: The effect of temperature of the medium for 2,4 CB biodegradation by the consortium was assessed. Highest percentage of growth was obtained at 35 °C (Fig.5 and 6).

Growth of 2,4 CB degrading consortium on different substrates: The consortium was able to utilize 2 CBA, 4

Table 1: Effect of different carbon source on the growth of and degradation of 2,4 CB by consortium with 100 ppm of 2,4 CB after 30 hrs (hour of incubation

S.No	Carbon source	Cell density as OD 600nm after 30hr	Chloride release
1	No cosubstrate	SMS medium	0.46mM
2	Glucose	0.4	0.10mM
3	Sodium glutamate	0.44	0.47mM
4	Mannitol	0.3	0.10mM
5	Maltose	0.282	0.12mM

Table 2: Effect of different concentrations of sodium glutamate on degradation of 2,4 CB by consortium (GSa and GSb) in SMS medium with 2,4 CB (100 ppm) after 30 hrs

S. No S. No	Carbon source in (%)	Cell density as OD (600 nm)	Chloride release in (mM)
1	0.1	0.38	0.28
2	0.2	0.48	0.49
3	0.3	0.44	0.3
4	0.4	0.28	ND (not detected)

Table 3: Effect of pH 7 on biodegradation of 2,4 CB by consortium in SMS medium with 2,4 CB (100 ppm) concentration

S.No S.No	Incubation in hr	Cell density as OD 600 nm	Chloride release in mM
1	10	0.03	0.05
2	20	0.3	0.25
3	30	0.5	0.3
4	40	0.53	0.45
5	50	0.53	0.45

Table 4: Effect of different pH on growth of consortium (GSa and GSb) with 2,4 CB as a substrate

Hour (600 nm)	pH 5	pH 6	pH 7	pH 8
0	0.011	0.017	0.017	0.011
5	0.055	0.024	0.032	0.011
10	0.014	0.024	0.15	0.011
15	0.056	0.1	0.3	0.011
20	0.07	0.25	0.47	0.02
25	0.12	0.39	0.46	0.02
30	0.123	0.42	0.45	0.03
35	0.15	0.422	0.45	0.03
40	0.15	0.41	0.452	0.03
45	0.15	0.399	0.452	0.03
50	0.149	0.398	0.452	0.03

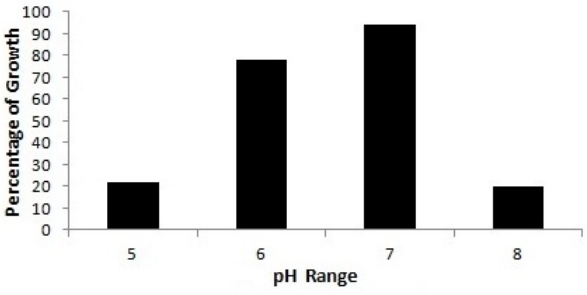

Figure 4:Percentage of growth at different pH

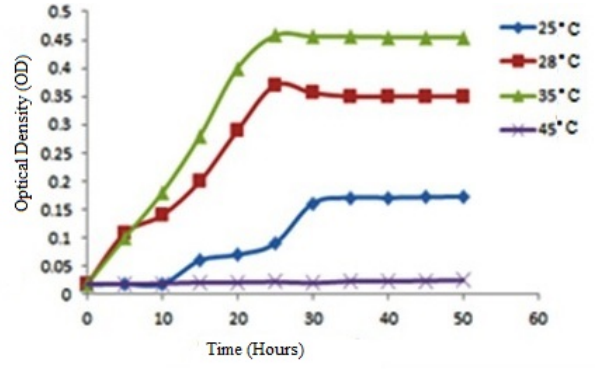

Figure 5:Optimization of growth at different temperature

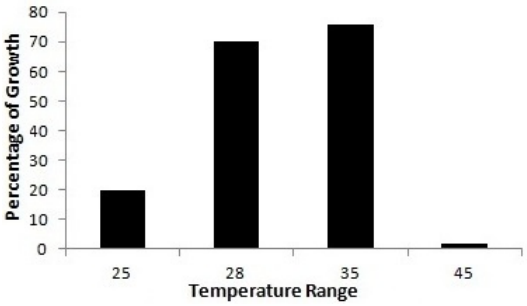

Figure 6:Percentage of growth at different temperature

CBA 2,4 CBA, Toluene, benzene (Fig. 7) in addition to 2,4 CB.

3.2 Determination of HOPDA activity

The culture filtrate of the consortium was analyzed using the media extract spectrophotometrically (350-450 nm), a characteristic biphenyl-derived product with maximum absorption of yellow biphenyl meta cleavage product (398 nm) formed during incubation with 2,4 CB (Table. 5).

Table 5: Time courses of the changes of 2-hydroxy-6oxo-6phenylhexa-2,4-dienoate (HOPDA) derived from 2,4 CB and absorption maximum of supernatant of culture medium inoculated with consortium with 2,4 CB substrate

Time (hr)	Cell density (400 nm)	Absorption at different wavelengths (350-450 nm)	Cell density (OD)
0	0.01	350	0.01
5	0.01	360	0.15
10	0.09	370	0.158
15	0.2	380	0.18
20	0.18	390	0.26
25	0.19	400	0.25
30	0.2	410	0.25
35	0.25	420	0.21
40	0.25	430	0.21
45	0.25	440	0.21
50	0.24	450	0.2

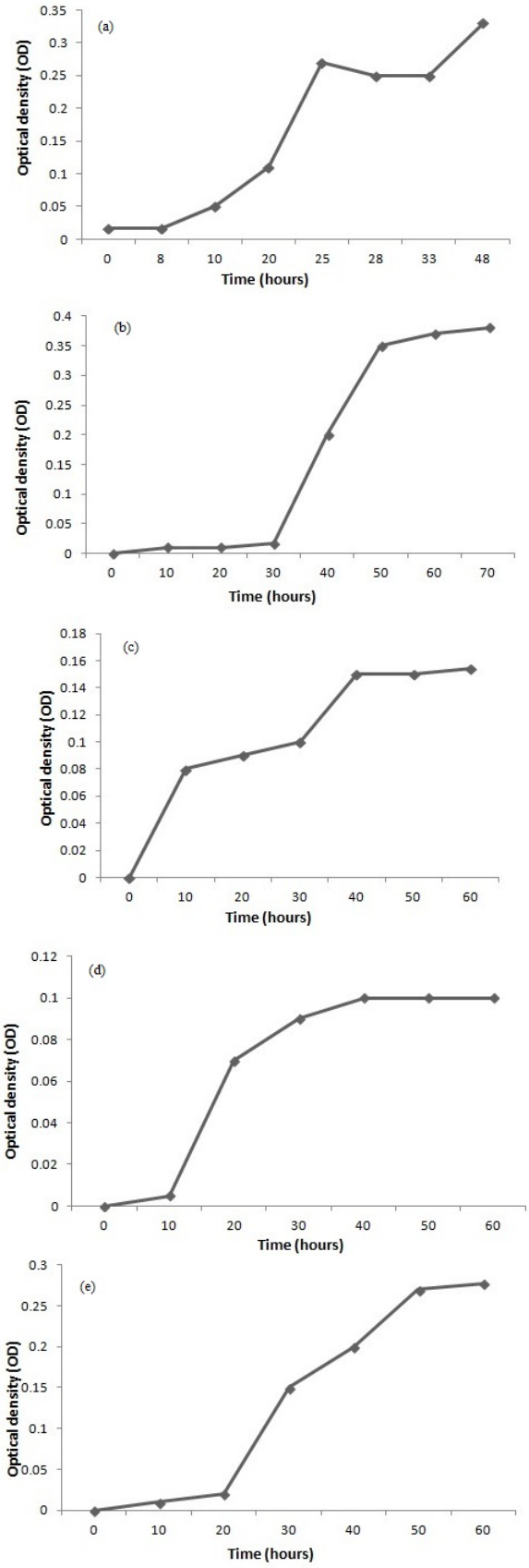

Figure 7: Growth Curve of consortium GSa and GSb on various substrates at 600 nm: (a) 4 CBA, (b)2 CBA, (c) 2,4 CBA, (d)Benzene, (e)Toluene

3.3 Enzyme assay

The cell free extract showed the presence of 2,4 dichlorobiphenyl oxidoreductase with specific activity of 0.3250 mol/min/mg after 25hr and 0.6790 mol/min/mg after 35hr. While, cell free extract showed negligible specific activity at 20 hr and 0.6118 mol/min/mg after 35 hr (Table 6). In addition, the cell free extract showed the presence of 2,4 dichlorobiphenyl oxidoreductase with specific activity of 0.3250 mol/min/mg after 25 hr and 0.6790 mol/min/mg after 35 hr. Similarly, the cell free extract showed presence of hydroxylase enzyme with specific activity of 0.6118 mol/min/mg after 35 hr (Table 6).

Table 6: Enzyme activity

Enzyme Activity	Time (Hrs)	Specific activity (mole/ml/mg)
2,4 dichlorobiphenyl Oxidoreductase	25	0.325
2,4 dichlorobiphenyl Oxidoreductase	35	0.679
Hydroxylase	20	Not detected
Hydroxylase	35	0.6118

4 Discussion

For maximum 2,4 Chlorobiphenyl (polychlorinated biphenyl (PCB) congener), degradation using *Pseudomonas* sp consortium GSa and GSb, various parameters were optimised. The low water solubility of PCB and their tendency to adhere tightly to soil are limiting factors for efficient PCB biodegradation. Therefore, an increase in the rate of solubilization and entry of PCB into the cell would accelerate degradation. One way to increase the rate of solubilization is to use surfactants. The assimilation of 2,4 CB was increased when surfactant Tween 80 was used, with 0.45 mM Chloride release, indicating the usefulness of the surfactant. This study was also supported by other research groups, where the microorganism degraded the substrate in the micelles which made available by the surfactant therefore the microorganism could biodegrade xenobiotic compound (Lajoie et al., 1994). Thus, the surfactant (Tween 80) enhanced the bioavailability of the substrates when consortium was used. Similar studies were also supported the enhancement of biodegradation by addition of surfactant (Pieper, 2005; Ron and Rosenberg, 2001). However, some surfactants promoting the solubilization of PCB did not necessarily increased the rate of PCB biodegradation (Billingsley et al., 1999) while, 2, 4 CB degradation was efficient with the usage of the surfactant. Microorganisms can be detrimentally affected if the initial concentration of the pollutant is very high. Although, the limit of tolerance to 2,4 CB was 130 ppm, maximum growth was at 90 ppm. In general, microorganisms grow mostly in a medium supplemented with additional substrate (Harder, 1982). Microorganisms acquire nutrients, energy and electrons from their environment. To support the growth, organic substrates provide energy and building materials that are used for the growth of new cells, cell maintenance and co-metabolism of other less degradable substances (Cornelissen and Sijm, 1996). According to Leach et al., (1994), the addition of compounds that act as co-metabolites is required to induce the requisite metabolic pathway or provide the necessary energy source for the cells to degrade a particular xenobiotic compound. Therefore, in the present study various carbon substrates such as glucose, maltose, mannitol, and sodium glutamate were used for maximum degradation of 2,4 CB. The culture media was supplemented with 2,4 CB, maximum chloride release of 0.47mM was observed with Sodium glutamate than other substrates and the concentration of sodium glutamate was also standardised for maximum degradation. At 0.2% of Sodium glutamate concentration maximum 0.49 mM Chloride release was observed, indicating additional source of carbon requirement at optimum concentration for better 2,4 CB degradation. Similarly, Loh and Wang (1998) reported that sodium glutamate enhanced xenobiotic degradation by stimulating cell viability and increasing toxicity tolerance of *Pseudomonas putida* ATCC 4951. These finding suggest that the additional carbon source, thus, may aid in reducing the toxicity and growth inhibition of xenobiotic on cells, thereby increasing the tolerance rates of xenobiotic. Conventional carbon source may also provide reducing power or degradation of recalcitrant compounds (Perkins et al., 1994) or in some cases act as inducing agents of biodegradation enzymes (Chaudhuri and Wiesmann, 1995). However in the present study glucose addition although promoted cells growth, did not increase chloride release indicating attack on non-substituted aromatic ring. Further mannitol and maltose also did not support the biodegradation effectively with increase in biomass and chloride release. Therefore, there is an agreement with Loh and Wang (1998) that the supplementation of glucose caused a significant drop in pH from 7.2 to 4.3 resulting in growth inhibition leading to reduction of phenol degradation. Highest degradation of phenol by Rhodococcus UKM-P (Suhaila et al., 2010) and Rhodococcus erythropolis was also showed highest degradation at 30^{o}C (Prieto et al. 2002). The degradation of 2,4 CB was maximum at 35^{o}C (Fig.6) indicating the relative thermal stability of the consortium. Further, highest 2,4 CB biodegradation with maximum chloride release was obtained at pH 7, and similar results were also found in biodegradation of phenol by Rhodococcus UKM-P (Suhaila et al., 2010). However, the consortium GSa and GSb showed to assimilate wide range of PCB congeners including Chlorobenzoic acid without inducer like biphenyl. Biphenyl could itself be toxic compound and often subject to regulatory mechanism (Komancova et al., 2003). In the present study, incubation of the consortium with biphenyl and 2,4 CB together decreased the biodegradation activity by the consortium GSa and GSb. These results were contrary to our observations, since biphenyl is mainly acts as an inducer in degradation of PCB compound (Kohler et al., 1998). In addition, different concentration of 2,4 CB was optimised where the consortium was tolerant upto

130 ppm, while, 90 ppm could be degraded efficiently (Fig.2). The consortium GSa and GSb degraded 2,4 CB, which was evident in growth studies, chloride release and disappearance of substrates which was measured spectrophotometrically. Further, the degradation of 2,4 CB by the consortium was optimum with additional carbon source Sodium glutamate (0.2%), surfactant at pH $\frac{7}{3}$ ° C/rpm- 130 and 100 ppm of 2,4 CB. Hence, isolated consortium GSa and GSb would be a promising candidate in-situ bioremediation after validation.

5 Conclusion

Pseudomonas spp (GSa and GSb) consortium was previously isolated and identified in our laboratory, was optimised for the degradation of 2, 4 chlorobiphenyl. For maximum degradation optimum physical conditions were evaluated. The maximum degradation was observed at 35ºC, pH 7, rpm-130, and the concentration of 2,4 CB was 90 ppm. Supplementation of 0.2% sodium glutamate and addition of surfactant Tween 80 resulted in highest amount of 2,4 CB degradation which was measured spectrophotometrically. In addition, the consortium showed their diverse degradation ability when different xenobiotic substances are used as carbon source. The results perhaps indicate applicability of the consortium for in-situ bioremediation, since soil would contain variety of xenobitocs.

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