STUDY OF CARBAZOLE DEGRADING MARINE AND TERRESTRIAL BACTERIAL ISOLATES FROM VALSAD DISTRICT

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Abstract : Carbazole is a nitrogen heterocyclic compound released into the environment, used as a chemical feed stock for the production of dyes, medicines and plastics. Carbazole and their derivatives are detected in atmospheric samples, river sediments, marine water and ground water. Enriched Mineral salts medium (MSM) broth was used to isolate bacteria able to use carbazole as a sole Carbon and Nitrogen and plate assay using MSM agar plate was developed to select carbazole degrading microorganisms. Ten different bacterial isolates were obtained which were capable of utilizing carbazole as the only sole source of carbon and nitrogen added. From the obtained ten isolates C:1, C:2 and C: 3 were from marine water samples, C:4, C:5 and C:6 from marine soil samples, C:7 and C:8 from Pond water samples and C:9, C:10 were from garage soil samples. All isolates were further studied for their "Bioavailability assay" by using different stress and favourable conditions, among all C: 2 isolate showed the maximum considerable and noticeable result in MSM medium. From the bioavailability assay results carbazole degradation studies was carried out using C: 2 isolate by adding 100 ppm and 500 ppm concentration of carbazole in MSM broth medium from which the concentration of carbazole reduced to 8.33 ppm and 50 ppm in 144 hours containing 100 ppm and 500 ppm respectively. Immobilization studies were carried by immobilizing C: 2 isolate in the form of calcium-alginate beads and PVA-alginate beads. Study of Mechanical stability of calcium-alginate and PVA-alginate beads was checked by sonication. PVA-alginate beads were more stable than calcium-alginate beads. Viability of the immobilized cells was tested by calculating colony forming units using both the types of beads, PVA-alginate beads shows more viability than calcium-alginate beads. Carbazole degradation studies with immobilized cells was done by taking absorbance OD_{600}, the concentration reduced to 41.66 ppm and 33.33 ppm in 144 hours using Calcium- alginate beads and PVA- alginate beads respectively containing 100 ppm concentration of carbazole and 241.6 ppm and 120.8 ppm respectively containing 500 ppm concentration of carbazole.

IndexTerms **- Bacteria, Carbazole, Bioavailability assay, Immobilization, Calcium alginate, Poly vinyl alcohol.**

1. INTRODUCTION

Carbazole is one of the recalcitrant nitrogen heterocyclic compound. It is very difficult to be degraded by most of the microorganisms in the environment. It is known to be toxic, teratogenic, mutagenic pollutant which is harmful to human health, chiefly found in the wastes generated by various pharmaceuticals and dye industries (Benedik *et al.*, 1998). Several bacteria have been reported to utilize carbazole viz. *Enterobacter, Gordonia, Klebsiealla, Pseudomonas, Sphingomonas* etc (Santos et al. 2006; Li et al. 2008; Larentis et al.2011; Singh *et al.,* 2011 a, 2011 b). They follow similar biochemical pathway and convert carbazole to antranilic acid which is then mineralized. Genes and enzymes involved in the conversion of carbazole to antranilic acid are also well established (Nojori *et al*., 2001). Bioremediation can be carried out by both free and immobilized cells but immobilized cell system shows several unique advantages over free cells such as prolonged activity and stability of the biocatalyst, feasibility of continous processing, increased tolerance to high substrate concentration, easier recovery with no need for separation and filteration steps, regeneration and reuse of the biocatalyst for extended periods in batch operations, reduction of microbial contamination risk ans ability to use smaller bioreactors with simplified process designs (Siripattanakul *et al*., 2008; Ahamad and Kunhi 2011). The key for immobilization is the choice of the support material. Each support has its own advantage and disadvantage in terms of the microorganism and the compound to be degraded. PVA is the most promising matrix as it is cheap, non toxic and mechanically robust. However, the biggest limitation with the use of PVA is the formation of sheets rather than beads resulting in the decrease of surface area. It has been reported that PVA beads are formed by the addition of sodium alginate along with PVA to the saturated boric acid and calcium chloride (Zain *et al*,. 2011). The release of carbazole into the environment from diverse anthropogenic sources is of serious health and environmental concern, as carbazole is both mutagenic and toxic and classified as benign tumorigen (Smith and Hansch 2000; Nojiri and Omori 2007). Carbazole is found in creosote, crude oil, shale oil and used as a feedstock for the manufacture of dyes plastics and medicines (Singh et al. 2011). Carbazoles are dominant as structural motifs in various synthetic materials and naturally occuring alkaloids (Roy *et al*., 2012). It is widely used as a model compound for the study of biodegradation of aromatic N-heterocyclic hydrocarbons. Carbazole directly affects the refining process by its conversion into basic derivatives during cracking, which allows it to adsorb to the active sites of the cracking catalyst (Benedik *et al*., 1998; Nojori and Omori 2007). The hydroxylated carbazole derivatives used in pharmaceutical industry are value-added substances exhibiting strong antioxidant activity and widely used in the treatment of encephalopathy, cardiopathy, hepatopathy and arteriosclerosis (Seto 1991). Furthermore, carbazole moiety is considered as one of the pharmacophores in the cardiovascular pharmacuticals cavedilol and carazolol, which are used in the treatment of hypertension, ischmic heart disease and congestive heart failure (Lateef *et al*., 2012).

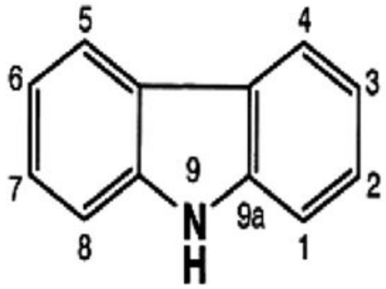

figure.1 Molecular structure of carbazole
source: (Nojori *et al*., 2001)

2. RESEARCH METHEDOLOGY

2.1 Chemicals

Carbazole was purchased from Kemphasol (Gujarat, India), Dimethyl sulfoxide (DMSO) was purchased from Qualikems (Gujarat, India).

2.2 Culture media

Mineral salts medium (MSM) used for carbazole degradation studies, composed of (per litre of solution) 2.44 g $KH^2PO_{4;}$ 1.65 g of K_2HPO4 ;0.2 g of $MgSO_4.7H_2O$; 0.003 g of $CaCl_2.2H_2O$; 0.02 g of NaCl; 1 g of KNO_3.

2.3 Enrichment and isolation of carbazole degrading bacterial isolates

For enrichment studies, marine water samples and marine soil samples, pond water samples and garage soil samples were collected from various places of Valsad district (Tithal beach, valsad), Nargol beach, Gujarat, Garage soil samples (Nargol, Gujarat), Pond water samples (Nargol, Gujarat). Enrichment experiments were performed in MSM broth containing 300 ppm of Carbazole as a sole source of carbon, nitrogen and energy. After enrichment, a loopful of enriched MSM medium was streaked on MSM agar plates were further incubated at Room temperature for 24 to 48 hours. The growths of different types of colonies were observed and were further purified and had been selected for bioavailability assay.

2.4 Bioavailability assay

The selected isolates were subjected to bioavailibility assay to check their effective carbazole utilization as both carbon and nitrogen source viz. M-I, M-II, M-III and M-IV. In M-I carbazole (300 ppm) was used as the only source of nitrogen with additional glucose (5g/litre) and glycerol (6.4ml/litre) as carbon sources; media M-II was taken as positive control with NH4Cl (2g/liter) as an alternative nitrogen source instead of carbazole. M-III was the test media containing carbazole (300 ppm) both as carbon and nitrogen source and lastly M-IV supplemented with carbazole but no isolates were added in it (negative control). All the isolates were firstly inoculated in MSM and after overnight incubation, 2% of fresh washed cells were inoculated in 100 ml Erlenmeyer flasks containing 20 ml of different MSM (Mineral salts media) separately incubated at room temperature and 180 rpm. Growth was monitored spectrophotomerically by measuring the optical density after every 24 hours upto 96 hours at 600 nm (OD_{600}) by using different M (I to IV) media. Isolate which would show good results in M-I compared to M-III, believe to utilize carbazole as only carbon and nitrogen source. The main aim is to search for a potential microorganism which would be able to break aromatic backbone of carbazole by using it as carbon and nitrogen source. (Bhatnagar *et al.*, 2015).

2.5 Carbazole degradation studies

Biodegradation studies by pure, selected and 24 hours growing bacterial isolates were performed in100 ml of MSM broth (pH 7.0) supplemented with carbazole [100 ppm and 500 ppm] as a sole source of carbon and nitrogen in 500 ml Erlenmeyer flask. All experiments were carried out at Room temperature on incubator shaker at 180 rpm. MSM broth was incubated and studied till 144 hours. Time course of carbazole degradation was obtained by analyzing $OD_{600.}$The effective carbazole degrading isolates had been selected for bioavailability assay.

2.6 Immobilization of CAR: 2 isolate

During immoblization studies potential isolate C:2 was grown in the Mineral salts medium containing carbazole (500 ppm) as a sole source of carbon and energy. Cells were harvested during late logarithmic phase (40 hours) by centrifugation at 6000 rpm for 10 min at 4^0 C. The pellet obtained was used for the immobilization. For immobilization in calcium alginate beads, alginate (2% w/v) was dissolved in boiling water and autoclaved at 121^0 C for 15 min. The bacterial pellet obtained was suspended in alginate solution (Room temperature), mixed by using vortex. This cells/alginate mixture was extruded drop by drop into a cold, sterile 0.2 M $CaCl_2$ solution through a syringe needle with needle. The beads were hardened in the $CaCl_2$ solution for 2 hours. Finally these beads were washed and were stored in distilled water at 40° C till further studies. For the immobilization in PVA-alginate beads, sterilized solution containing PVA and sodium alginate was prepared by dissolving the compounds in boiling water and autoclaving at 121^0 C for 15 min. The bacterial pellet obtained was suspended in PVA-alginate solution, and mixed by mild vortexing. The above solution was extruded through a syringe into a beaker containing the gelling solution with known concentration. The beads were washed with distilled water to remove any gelling solution; finally these beads were stored with sterile distilled water at 4^0 C till further studies. (Bhatnagar *et al*., 2015).

2.7 Mechanical stability of beads

To determine the mechanical stability of beads prepared for this study, 50 and 10 beads of each type were taken seperately in 50 ml beakers with 10 ml distilled water and were sonicated at 20 KHz/min for three cycles and 20 KHz/min for six cycles. From these two different cycles beads were collected and used for mechanical stress study.

2.8 Tests for viability of immobilized cells

The viability of cells immobilized in Calcium alginate beads and PVA-alginate beads (10 and 50 beads each) were washed with sterile distilled water and disintegrated in 10 ml of buffer solution containing 0.05 M Na_2CO_3 and 0.02 M citric acid . The buffer solution (0.1 ml) was plated onto Mineral salts medium agar plates and CFU was calculated after incubating the plates at Room temperature for 16 hours. (Bhatnagar *et al*., 2015)

2.9 Carbazole degradation studies by immobilized cells

Based on the characterization results beads prepared using 6% PVA and 2% sodium alginate were used for immobilizing C:2 isolate. Carbazole degrading efficiency was assesed in 250 ml flask with 50 ml media (Mineral salts medium) containing desired amount of carbazole 100 ppm and 500 ppm. The beads were added to the flask and incubated in orbital shaker at Room temperature and 180 rpm. Samples of the medium were collected after every 24 hours, up to 144 hours and absorbance OD_{600} were taken spectrophotometrically.

3. RESULTS AND DISCUSSION

In this study, the biodegradation of cabazole by microorganisms isolated from marine water samples, marine soil samples, garage soil samples and pond water samples was studied. The result on biodegradation was depicted as concentration of residual carbazole with the time.

3.1 Enrichment and Isolation of bacteria

The enrichment of soil samples and water samples for carbazole degrading bacteria was carried out as mentioned above. Ten different bacterial isolates were isolated by enrichment culture using Mineral salts media supplemented with 300 ppm of carbazole as a sole source of carbon and nitrogen and were designated consequently starting from C:1 to C:10. Isolate C:1 was found to be Gram negative, short rods, occurring singly and in clusters, motile organisms. Isolate C:2 was found to be Gram positive, short rods, occurring singly and in clusters, C:3 was cocco- bacilli, occurring in clusters, C: 4 was Gram positive, cocci, occurring singly and in clusters, C:5 was Gram positive, cocci , occuring in clusters, C:6 was Gram positive, cocci occuring in clusters, C:7 was Gram positive cocci, occurring in clusters, C:8 was cocco- bacilli, occurring in clusters, C:9 was cocco- baclli occurring in clusters and C:10 was Gram negative, short rods, occurring singly.

3.2 Results of Bioavailability assay.

Bioavailability assay was carried out using all ten isolates, carbazole degradation activity of all ten isolates was checked after every 24 hours by measuring the absorbance OD_{600}. Among all isolate C:2 was showing maximum absorbance values. Maximum growth and maximum utilization of carbazole by the same isolate indicated that carbazole is being utilized for the growth of the bacteria as both carbon and nitrogen source. Utilization of pollutant carbazole as both carbon and nitrogen source is a prequisitive for successful bioremediation process. it was noted that all the ten isolates could variably grow in M-I, M-II and M-III but Isolate C:2 showed comparatively best results to others (Figure: 1). No growth was observed in M-IV (Negative control) and noticeable growth of C:2 in M-III (Carbazole as carbon and nitrogen source) suggested that carbon and nitrogen is required for the growth of the isolates and they could efficiently utilize carbon and nitrogen present in pollutant carbazole.

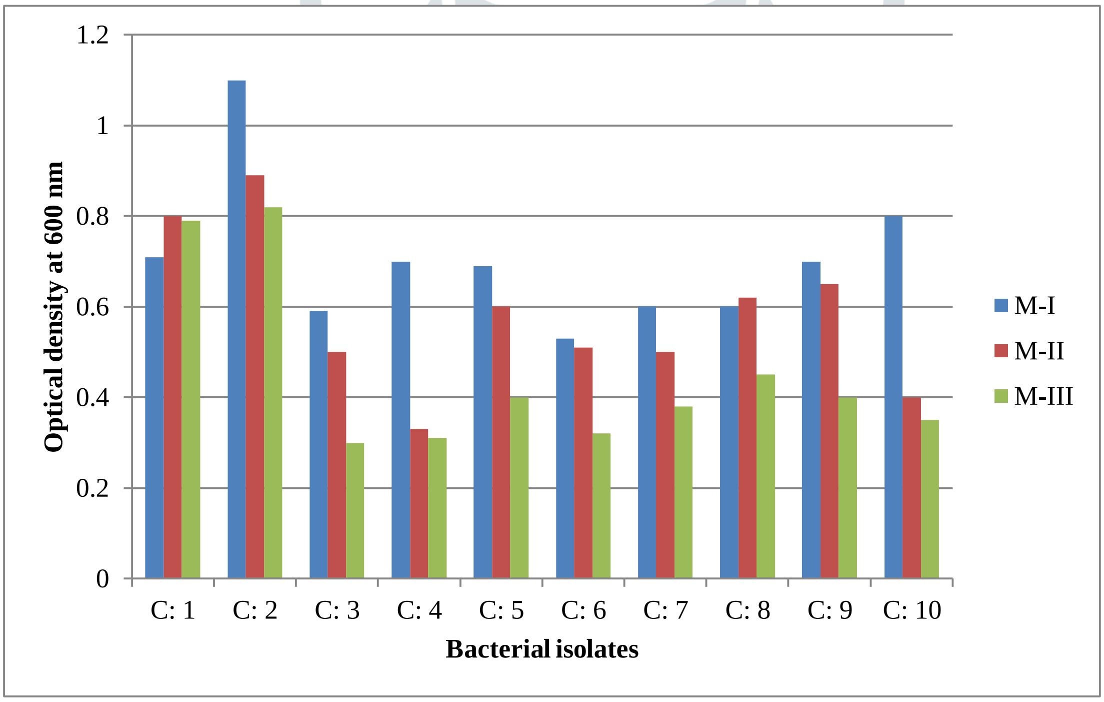

figure: 2 Spectrophotometric observations at 600 nm for all isolates after 96 hours in different mineral salts media. no growth was observed in media M-IV (negative control), where M-I is MSM with carbazole as only nitrogen source with additional carbon sources, M-II is MSM with ammonium chloride as an alternative nitrogen source (no carbazole), M-III as MSM with only carbazole as a sole source of carbon and nitrogen.

3.3 Results of Carbazole degradation studies

The degradation of carbazole by the growing cells of isolate C:2 was studied by supplementing 100 ppm and 500 ppm of carbazole in 100 ml of sterile MSM broth medium for 144 hours. The optical density OD_{600} decreases after every 24 hours of incubation time. The concentration reduced to 8.33 ppm in the MSM broth containing 100 ppm of carbazole and 500 ppm in the MSM broth containing 500ppm of carbazole.

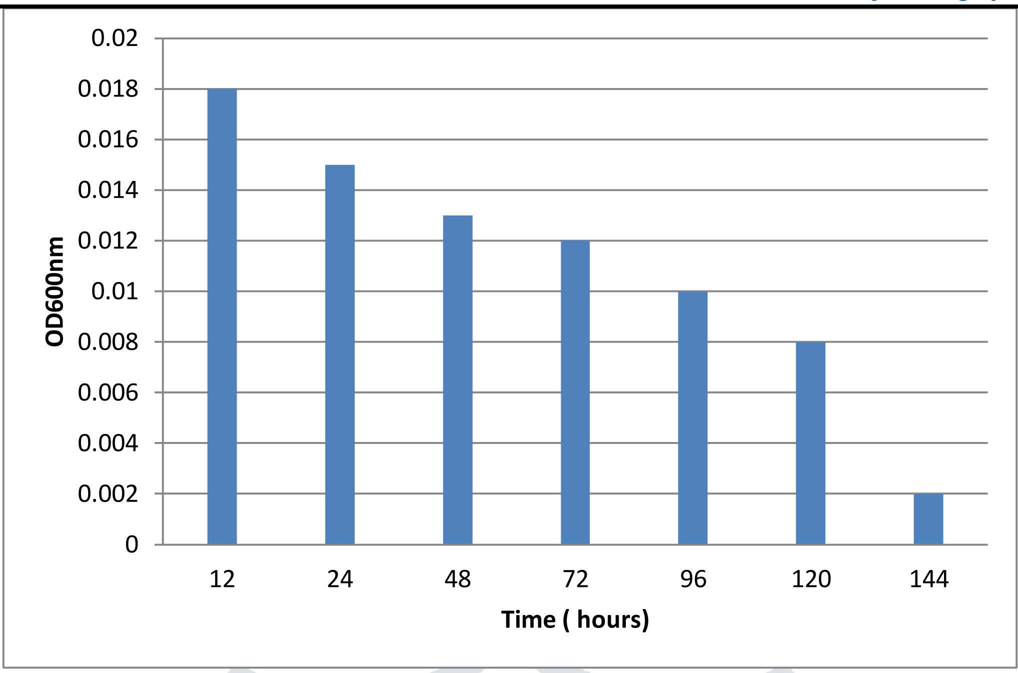

figure:3[A] showing spectrophotometric observations at 600 nm for isolate C:2 cultured in MSM broth containing 100 ppm concentration of carbazole.

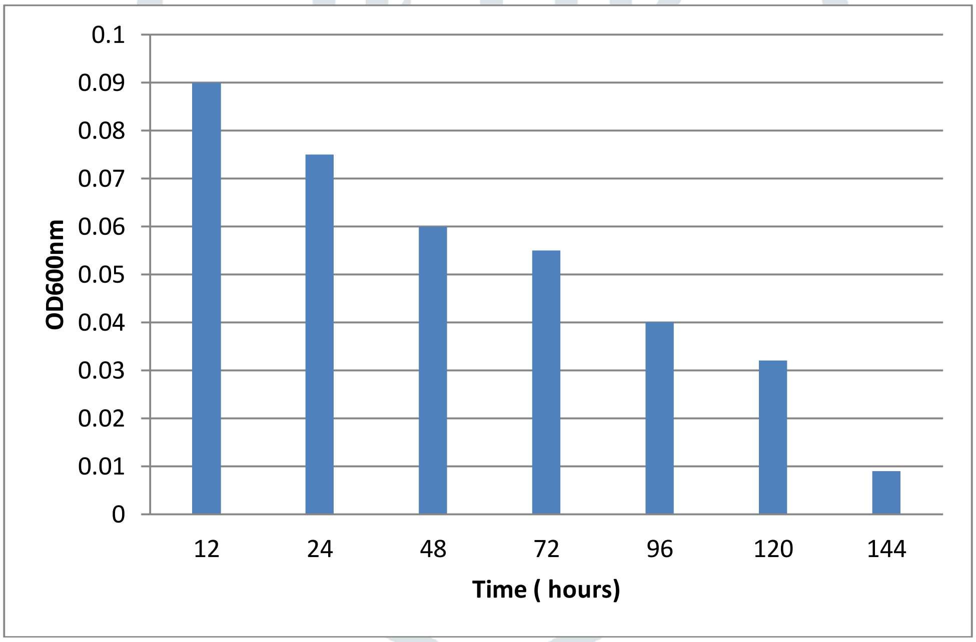

figure: 3[B] showing spectrophotometric observations at 600 nm for isolate C:2 cultured in MSM broth containing 500 ppm concentration of carbazole.

3.4 Results of mechanical stability of beads.

The most suitable bead type was initially screened based on the mechanical stability of Calcium-alginate and PVA-alginate beads. Both the different types of beads showed equal stability and were stable after the sonication of 3 and 6 minutes and the Viabilty of the immobilized cells. Both the different types of beads were taken for checking the number of viable cells immobilized inside the beads. The stability of both the types of beads was almost similar. PVA-alginate beads gave more counts of CFUs in comparision to Calcium-alginate beads.

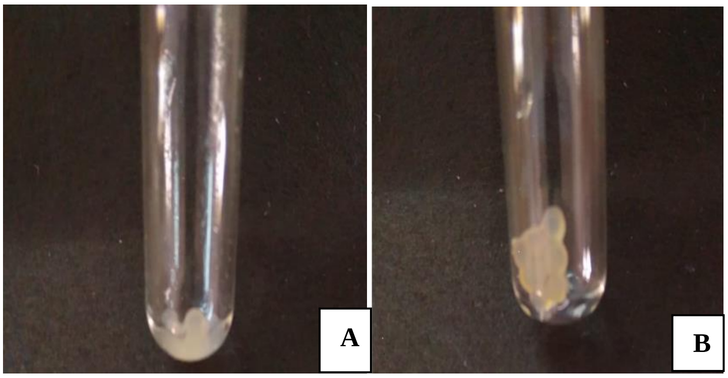

FIGURE 4 [A]: SHOWS PVA- ALGINATE BEADS BEFORE SONICATION

FIGURE 4 [B]: SHOWS CALCIUM- ALGINATE BEADS BEFORE SONICATION

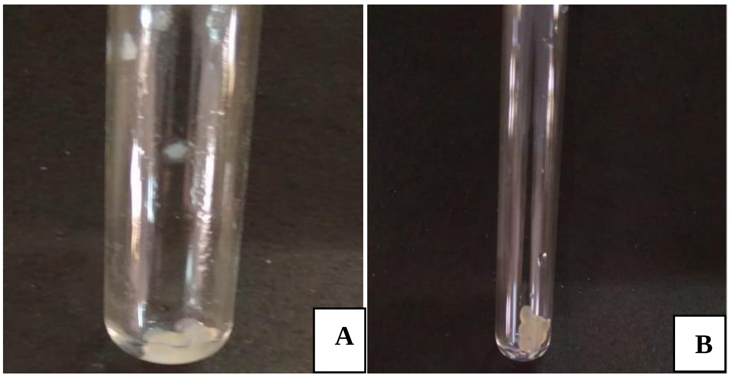

FIGURE 5 [A]: SHOWS PVA- ALGINATE BEADS AFTER SONICATION

FIGURE 5 [B]: SHOWS CALCIUM- ALGINATE BEADS AFTER SONICATION

3.5 Results of Tests for viability of immobilized cells

The effect of different concentrations of carbazole i.e. 100 ppm and 500 ppm was also studied on the degradation activity by counting the no of CFUs by immobilized cells in PVA – alginate and Calcium- alginate beads. The cells immobilized in PVA- alginate beads were more viable than Calcium- alginate beads. 298 no CFUs were found after plating 0.1 ml of dissolved buffer solution containing dissolved PVA- alginate beads after 24 hours of incubation and 198 no of CFUs were found after plating 0.1 ml of dissolved buffer solution containing Calcium alginate beads after 24 hours of incubation time. The more no of colony forming units indicates more no of viable cells.

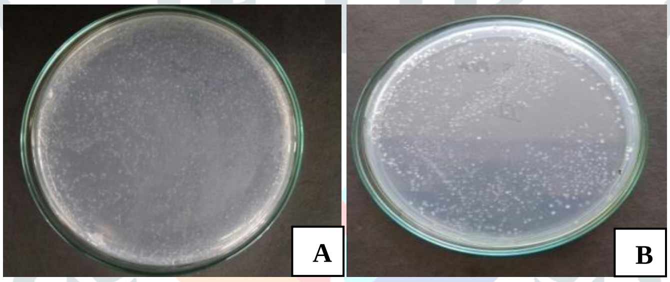

figure : 6 [A] No of CFUs observed by plating dissolved PVA- alginate beads

figure : 6 [B] No of CFUs observed by plating dissolved Calcium- alginate beads

3.6 Results of Carbazole degradation studies by immobilization of C:2 isolate in Calcium- alginate beads and PVA- alginate beads.

The degradation of carbazole was studied by immobilizing isolate C:2 in the form of Calcium- alginate and PVA- alginate beads by supplementing MSM broth medium with 100 ppm and 500 ppm concentration of carbazole. The optical density reduced after every 24 hours of incubation. The concentration reduced to 33.33 ppm and 41.66 ppm using PVA- alginate and Calcium- alginate beads after 144 hours in MSM broth medium containing 100 ppm concentration of carbazole and 120.8 ppm and 241.6 ppm containing 500 ppm concentration of carbazole.

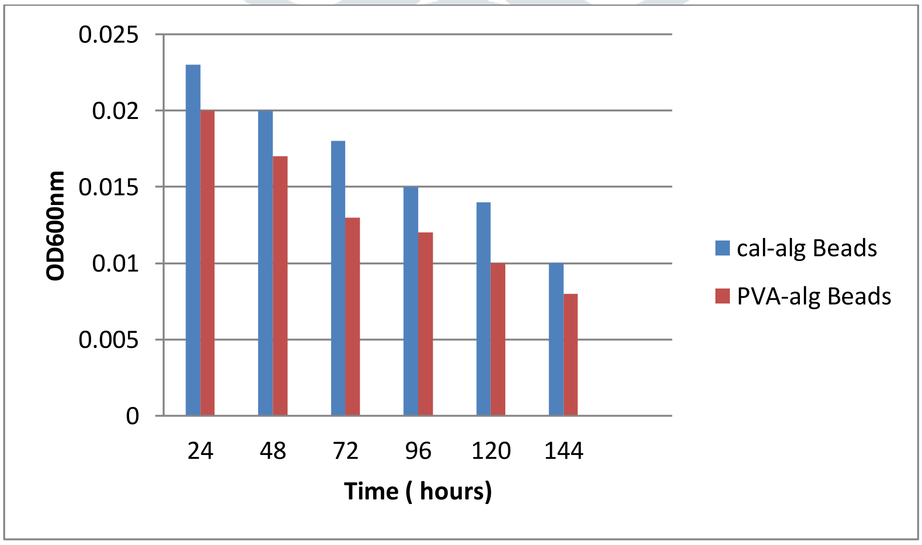

figure: 7[A]showing spectrophotometric observations at 600 nm by using PVA- alginate and Calcium- alginate beads added in MSM broth medium supplemented with 100 ppm of carbazole.

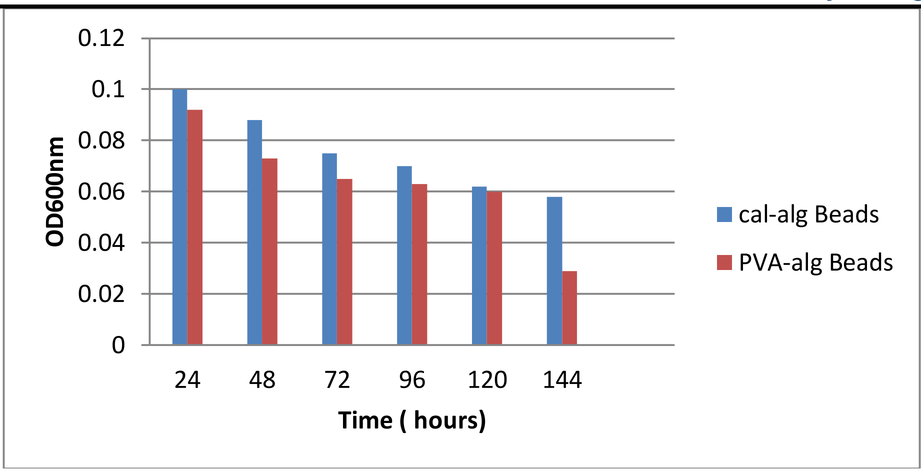

figure: 7[B]showing spectrophotometric observations at 600 nm by using PVA- alginate and Calcium- alginate beads added in MSM broth medium supplemented with 500 ppm of carbazole.

4. ACKNOWLEDGMENT

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REFERENCES

[1] Ahmad, A. B., Zulkharnain, A. B., & Husaini, A. A. S. B. A. Substrate specificity of angular dioxygenase from carbazole-degrading bacterium Neptuniibacter sp. strain CAR-SF.

[2] Bressler, D. C., & Fedorak, P. M. (2000). Bacterial metabolism of fluorene, dibenzofuran, dibenzothiophene, and carbazole. *Canadian journal of microbiology*, *46*(5), 397-409

[3] Dumeignil, F. (2008). Degradation of carbazole by Novosphingobium sp. strain NIY3. *Journal of the Japan Petroleum Institute*, *51*(3), 174-179

[4] Evaluation of carbazole degradation by Enterobacter sp. isolated from hydrocarbon contaminated soil. *Recent Research in Science and Technology*, *3*(11).

[5] Fuse, H., Takimura, O., Murakami, K., Inoue, H., & Yamaoka, Y. (2003). Degradation of chlorinated biphenyl, dibenzofuranand dibenzo-p-dioxin by marine bacteria that degrade biphenyl, carbazole, or dibenzofuran. *Bioscienc*

[6] Gai, Z., Yu, B., Li, L., Wang, Y., Ma, C., Feng, J., ... & Xu, P. (2007). Cometabolic degradation of dibenzofuran and

[7] Larentis, A. L., Sampaio, H. C. C., Carneiro, C. C., Martins, O. B., & Alves, T. L. M. (2011). Evaluation of growth dibenzothiophene by a newly isolated carbazole-degrading Sphingomonas sp. strain. *Appl. Environ. Microbiol.*, *73*(9), 2832-2838. Singh, G. B., Srivastava, S., Gupta, S., & Gupta, N. (2011).

[8] Inoue, K., Habe, H., Yamane, H., Omori, T., & Nojiri, H. (2005). Diversity of carbazole-degrading bacteria having the h, carbazole biodegradation and anthranilic acid production by Pseudomonas stutzeri. *Brazilian Journal of Chemical* car gene cluster: isolation of a novel gram-positive carbazole-degrading bacterium. *FEMS microbiology letters*, *245*(1), *Engineering*, *28*(1), 37-44. 145-153.

[9] Li, L., Li, Q., Li, F., Shi, Q., Yu, B., Liu, F., & Xu, P. (2006). Degradation of carbazole and its derivatives by a bacteria. *Curr Res Technol Educ Top Appl Microbiol Microb Biotechnol*, *2*, 1311-1321.

[10] Maeda, R., Ito, Y., Iwata, K., & Omori, T. (2010). Comparison of marine and terrestrial carbazole-degrading Pseudomonas sp. *Applied microbiology and biotechnology*, *73*(4), 941. [10] Ouchiyama, N., Zhang, Y., Omori, T., &

[11] Kodama, T. (1993). Biodegradation of carbazole by Pseudomonas spp. CA06 and CA10. *Bioscience, biotechnology, and biochemistry*, *57*(3), 455-460.

[12] Salam, L. B., Ilori, M. O., & Amund, O. O. (2015). Carbazole degradation in the soil microcosm by tropical bacterial strains. *Brazilian Journal of Microbiology*, *46*(4), 1037-1044.

[13] Shi, S., Qu, Y., Ma, F., & Zhou, J. (2014). Bioremediation of coking wastewater containing carbazole, dibenzofuran and dibenzothiphene by immobilized naphthalene-cultivated Arthrobacter sp. W1 in magnetic gellan gum. *Bioresource technology*, *166*, 79-86.

[14] Singh, G. B., Srivastava, S., & Gupta, N. (2010). Biodegradation of carbazole by a promising gram-negative bacterial strains. *Brazilian Journal of Microbiology*, *46*(4), 1037-1044.bacterium. *World Acad Sci Eng Technol*, *70*, 784-787.

[15] Shotbolt-Brown, J., Hunter, D. W., & Aislabie, J. (1996). Isolation and description of carbazole-degrading bacteria. *Canadian journal of microbiology*, *42*(1), 79-82.

[16] Singh, G. B. (2013). Microbial screening and expression of gene involved in carbazole degradation.