Biodegradation of phenol using No cardia hydro carbon oxydans, Saccharomyce cerevisiae and Candida slooffi microorganisms immobilized on glass balls– A comparative study

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Abstract

Phenol is one of the important chemical constituents which is highly used for the production of numerous chemicals and value-added products. The huge application of phenol leads to its enormous presence in the environment as a toxic effluent. The present work emphasizes on the biodegradation of synthetic phenol using glass beads immobilized with *Nocardia Hydro carbon oxydan* biomass. The study also emphasizes on the performance of packed bed bioreactor on the degradation process. Experimental studies reveals that the microorganisms are able to degrade phenol at a lower inlet concentration of 300 ppm. The effect of the quantity of the immobilized carrier particles on the phenol degradation was studied. The results obtained justifies the optimum type of bioreactor and type and condition of biomass which can be used for best possible phenol removal from effluent waters.

Keywords: *Phenol; biodegradation; biomass; microorganisms*

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1. Introduction

Water is an indispensable natural resource on this earth on which all life depends [1]. Next to air, the other important requirement for human life to exist is water. Water is available in various forms such as rivers, lake, streams etc. Although water is very abundant on this earth, yet it is very precious [2]. So there is a great need to manage water resources in a sustainable, efficient and effective manner to ensure long-term availability for use [3]·Pollution of water resources is major concern today [1,4]. The natural water bodies are contaminated by various toxic pollutants [5] and organic compounds creating a major problem [6]. The long term consequence of these toxic effluents can cause nervous damage, cancer, malformation in urban children, mutagenic changes, and neurological disorder [7].

Nevertheless, to ensure sustainable quality of life the environmental impact of these activities must be minimized [8].Phenol has a huge application for the production of disinfectants, slimicidesand for medicinal preparations like nose drops, mouthwashes etc. It also has a good germicidal effect and hence can be effectively used in shaving cream. It is also used as peptizing agent and effective extracting solvent in refinery and lubricant production and as a primary petrochemical intermediate. Phenol is a major pollutant included in the list of EPA (1979). Phenol is toxic even at low concentrations and the toxicity of phenols for microbial cells has been investigated [9].Therefore, the environmentally unacceptable pollution effects of the wastes have been reported worldwide and the adverse effects of phenol on health are well documented [10].

Although a lot of work is done on bio degradation of phenol, the research work is not gone to industrial trails. It is may be because the biomass which is selected is mostly proprietary. The present work deals with shake flask studies to determine biomass growth rates and degradation rates of synthetic phenol and the study of performance of packed bed bioreactor on the biodegradation of phenol in synthetically prepared phenolic wastewater using glass beads

immobilized with *No cardia Hydro carbon oxydans.*

2. Materials and Method

2.1. Materials

Various Materials and Chemicals used

Analytical phenol from Merck India Ltd, Mumbai, India was used for the purpose. To test the phenol concentrations in samples and to generate calibration curve, color development is essential. For this chemical like ammonium chloride, ammonium hydroxide, potassium ferro-cyanide,4-amino antipyrine were used (Table 1). For the initial sub culturing and immobilization of microorganism agar and beef extracts along with mineral media chemicals like ammonium nitrate, ammonium sulphate, sodium chloride, di-potassium hydrogen ortho phosphate, potassium di-hydrogen ortho phosphate, ferrous sulphate, calcium chloride and magnesium sulphate were used.

Table 1. Chemicals and their source

S.No	Name of the Chemical	Source	Grade
1	4-Amino antipyrine	Central Drug House, Delhi	AR
2	Sodium chloride	Nice Chemicals	LR
3	G Glass beads	Quali Chemicals, Mumbai	LR
4	Agar-agar	Nice Chemicals	LR
5	Beef extract	Nice Chemicals	LR
6	NaOH	Finar Chemicals	AR
7	Potassium ferricyanide	Merck India	AR
8	Phenol	Merck India	AR
9	Ammonium chloride	Fisher Chemical	LR
10	Ammonium hydroxide	Merck India	AR
11	Ammonium nitrate	Fisher Chemical	LR
12	Ammonium sulphate	Fisher Chemical	LR
13	Di-Potassium hydrogen ortho phosphate	Finar Chemicals	LR
14	Potassium di-hydrogen ortho phosphate	Finar Chemicals	LR
15	Ferrous sulphate	Merck India	AR
16	Calcium chloride	Nice Chemicals	LR
17	Magnesium sulphate	Nice Chemicals	LR

Microorganisms used

The different microorganisms used for the process and their characteristics are shown in Table 2

No cardia hydrocarbon oxydans was obtained from National Chemical Laboratory, Pune.

Table 2. Characteristics of the *No cardia hydro carbon oxydans*

Kingdom	Bacteria (eubacteria) Terra bacteria group	Family	*Pseudomonadaceae*
Phylum	*Actinobacteria*	Genus	*Pseudo nocardia*
Subphylum	*Actinobacteria Gram-positive bacteria*	Species	*Pseudonocardiahydrocarbonoxydans*
Order	*Pseudonocardiales*		

2.2. Methods

Preparation of media

In the present study phenol was used as a nutrient and sole carbon source for the growth of organism as defined in the medium [11,12] and shown in the Table 3. To prevent precipitation of Ca^{++} and Mg^{++} the medium was prepared as two solutions A & B which were separately steam sterilized. The solution was adjusted to pH 7.0 by 0.1N NaOH.

Table 3. Mineral medium composition

	Chemicals	**Grams per litre**
Solution "A"	Ammonium nitrate	1.00
	Ammonium sulphate	0.50
	Sodium chloride	0.50
	Di-potassium hydrogen ortho phosphate	1.50
	Potassium di-hydrogen ortho phosphate	0.5
	Ferrous sulphate	0.002
Solution "B"	Calcium chloride	0.01
	Magnesium sulphate	0.50

Acclimatization procedure

The first step of the experiment is adaptation of microorganism [13,14] to the phenol substrate. About 99ml of mineral media were taken in the conical flask and sterilized in autoclave at 1.5Kg/cm^2 at 121^{o}C for 20min and cooled to room temperature. To this 1 ml of 10000 ppm phenol solution was added to get an initial phenol concentration of 100ppm and the total volume of 100ml.Aloop full of test organism obtained from the freshly sub cultured slant was inoculated into the above medium. The culture was incubated in a shaker for 48hr. This formed the primary acclimatized culture. The secondary acclimatized inoculum was prepared in the same way where in 1ml of primary culture was used instead of the subculture from the slant to inoculate the medium [15]. This was continued for the successive acclimatization and the acclimatized culture was stored in the refrigerator. The culture was acclimatized for 100 ppm four times. Then for the acclimatization of higher concentration of 200 ppm, 300 ppm, 500 ppm, 800 ppm and 1000 ppm were done successively following the above procedure. Acclimatized cultures were stored in incubator at 4^{o}C.

Cell immobilization to glass beads

Cell suspensions cultured for 48hr with fourth acclimatized inoculums were prepared. To every gram of glass beads 5 ml of cell suspension was added and it was refrigerated at 4^{o}C for 2 days. The immobilized glass beads was then filtered, washed and used in the experiment [16 - 18].

Preparation of phenol solution

10000ppm phenol solution stock was prepared by dissolving 5 g of phenol in distilled water to make up to 500 ml of solution.

Reagents preparation

Different reagents and their concentrations used for the analysis are shown in Table 4.

Table 4. Reagents for phenol analysis

Reagents	Concentration
Ammonium chloride solution	20.0g/l
Ammonium hydroxide solution	0.91sp.gr
Potassium Ferro-cyanide solution	80.0 g/l
4-Amino antipyrine solution	20.0 g/l

3. Experimental procedure

The bio mass is initially sub cultured, acclimatized and immobilized on glass beads. Required phenol solution along with mineral media is filled in the supply tank. The packed bed column was cleaned thoroughly with water and kept for drying. After drying, a known amount (200 g, 400 g, 600 g) of the immobilized glass beads were filled in the column. The active section of the column was filled completely with the phenol solution up to sampling probe which is 18 cm from the bottom of the column (Figure 1). Compressed air was sent from the bottom of the column for aeration at a constant rate to ensure oxygen concentration of >3 mg/lit in the reactor liquid. Synthetic wastewater containing phenol and mineral media solution was sent with a constant flow rate using peristaltic pump through the inlet at the bottom of the column. The effluent was collected from the probe 8 cm from the bottom of the column at a time interval of 4 hr and analysed for the phenol concentration, until the system reached steady state. Steady state conditions were assumed when phenol concentration in the effluent reached constant value. Then the beads were taken out and the film thickness and total amount of bio mass is estimated. Glass beads are washed several times with distilled water, sterilized and used again. Bio degradation is strongly a function of amount of substrate availability. Therefore effect of influent concentration and influent flow rates are studied. The amount of catalyst is one of the important parameter hence amount of bio mass in bio reactor is one of the important constituents that effects it. For this purpose amount of surface area provided for the bacterial growth is important. Even though temperature is one of the parameter of reaction, but growth of organism is retarded by temperature variations. Maintaining higher air flow rates are always advisable to operate, air flow rates are adjusted to see that DO is not decreased below 5ppm.

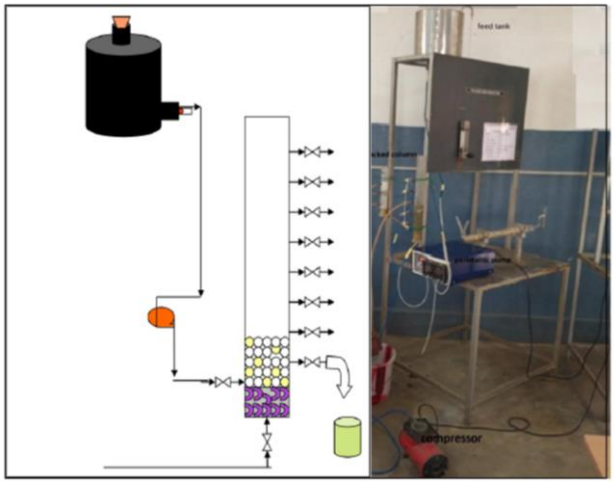

Fig. 1. Flow diagram of experimental setup

4. Results and Discussion

The present study was designed to observe the biodegradation of phenol using microorganism such as *No cardia hydro carbon oxydans*, *Saccharomyce cerevisiae* and *Candida slooffi*. A packed bed bioreactor having immobilized cells on glass beads as carrier particles has been selected to analyze the biodegradation of phenol.

4.1. Factors affecting phenol biodegradation by immobilized No cardia hydro carbon oxydans

Factors, such as the influent and effluent phenol concentration, quantity of carrier particles, free cell concentration, biomass dry weight etc., influences the phenol biodegradation during start up and at steady state in packed bed bioreactor. The effluent was collected at specific time intervals (0-84 hr) from bioreactor, and was analyzed for phenol concentration to evaluate the biodegradation of phenol in *No cardia hydro carbon oxydans* immobilized glass beads placed in a packed bed bioreactor. The number of carrier particles used was kept constant at about 6400 to analyze the effect of influent phenol concentration on effluent phenol concentration, free cell concentration and steady state.

4.1.1 Influent Phenol Concentration

Effect of influent phenol concentration on effluent phenol concentration during Start up

The influent phenol concentration at the start up was maintained between 200 and 1000 ppm. The decrease in effluent phenol concentration was observed from 4 hr in all groups at the start up phase (Figure 2). After 32 hr, the phenol concentration reduced from 200 ppm to 0.813 ppm. Similarly for 300 ppm and 500 ppm of initial phenol, the lowest effluent phenol concentration was obtained at 68 hr and 72 hr, respectively, whereas for 800 ppm and 1000 ppm, it was obtained at 68 hr of continuous biodegradation. The lowest concentration of phenol in these groups varied from 0.813 ppm to 416 ppm suggesting that 100% degradation was possible at 200 ppm while only 42% was achieved at 1000 ppm. A steady level of effluent phenol was observed after 68 hr in 800-1000 ppm phenol. The increase in initial phenol concentration was found to be accompanied by an increase in the time period (hr) to obtain lowest and steady effluent phenol concentration. The results indicate that the high influent phenol concentration was accompanied with lower rate of phenol degradation.

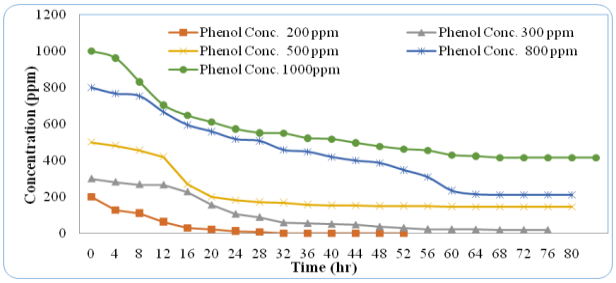

Fig. 2. Effect of influent phenol concentration on effluent phenol concentration during Start up in a packed bed bioreactor with No cardia hydro carbon oxydans immobilized on glass beads

***Conditions**: Initial phenol concentration: 500 ppm (a) Carrier particle size: 3 mm (b) Constant influent flow rate: 400 ml/hr*

Effect of influent concentration on free cell concentration during start up

Figure 3 represents the effect of initial phenol concentrations of 200-1000 ppm on the free cell concentration during start up and steady state in the packed bed bioreactor with immobilized No cardia hydro carbon oxydans. The increase in effluent free cell concentration of 200 and 300 ppm initial phenol concentration was up to a time period of 24 hr and 32 hr respectively during startup phase and followed by the decrease to 25.27 and 28.25 of free cell concentration at 40 hr and 64 hr respectively. The increase in free cells in 56 hr was up to 9.99 for 800 ppm and 6.81 free cells for 1000 ppm initial phenol concentration, while it decreased to 7.27 free cells at 68 hr for 800 ppm and 0.46 free cells at 72hr for 1000 ppm of initial phenol concentration. The effluent from 500 ppm phenol showed increase in free cell concentration during start up and reached to steady state having 39.55 free cells at 64 hr. The results showed that highest free cells concentration was found in effluent from 500 ppm at steady state among the 200-1000 ppm initial phenol concentrations. The lowest concentration of free cells (0.46) for 1000 ppm indicates the toxic effect of high phenol concentration of free cells. The low level of free cells during start up phase indicates high quantity of the immobilized cell available for phenol biodegradation while with increase in time the level of free cells also increase due to depletion of nutrition and cell lysis. However a decrease in free cells and constant effluent phenol at steady state indicate that the immobilized cells have utilized phenol as a nutrient source therefore reduced the toxic effect of phenol, whereas the low concentrations of free cell at steady state (7.27 and 0.46) for initial phenol concentration of 800 ppm and 1000 ppm, respectively, may be due to the detrimental effect of high phenol concentration on immobilized and free cells causing cell lysis

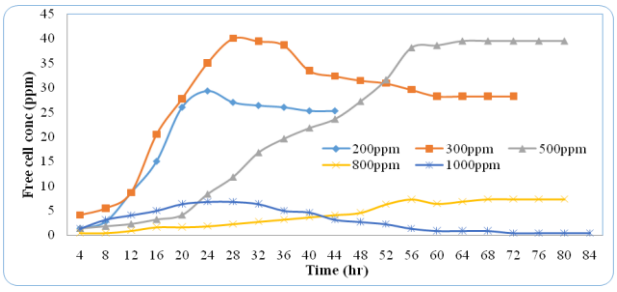

Fig. 3.Effect of influent phenol concentration on free cell concentration during Start up in a packed bed bioreactor with Nocardiahydrocarbonoxydans immobilized on glass beads

Conditions: Initial phenol concentration: 1000 ppm (a) Carrier particles size: 3 mm (b) Constant dilution rate: 0.0038547 hr^{-1}

4.1.2 Quantity of immobilized carrier particles

Effect of quantity of immobilized carrier particles used on effluent phenol concentration

(a) Initial phenol concentration: 1000 ppm

Figure 4 represents the initial phenol concentration of 1000 ppm and 6400-25600 carrier particles for time period of 0-84hr was used to evaluate the effect of quantity of immobilized carrier particles on effluent phenol concentration during start up in a packed bed bioreactor with immobilized *Nocardiahydrocarbonoxydans*. During start up phase decline in effluent phenol concentration was observed from 4 hr in 6400-25600 carrier particles, highest decline from 1000 ppm to 750 ppm phenol concentration for 25600 carrier particles was observed. On the other hand 6400 carrier particles showed the least alteration in phenol concentration from 1000 ppm to 962 ppm. This indicates high rate of phenol degradation in the presence of high quantity of carrier particles in the packed bed bioreactor. At 68 hr, 416 ppm effluent phenol obtained from 6400 carrier particles packed bed bioreactor, whereas effluent phenol concentration of 302 ppm and 97 ppm obtained at 48 hr for 12800 carrier particles and 25600 carrier particles, respectively and it was 165 ppm at 44 hr for 19200 carrier particles. The immobilized cell packed bed bioreactor having high carrier particles required less time to attain steady state, which suggests highly efficient phenol biodegradation.

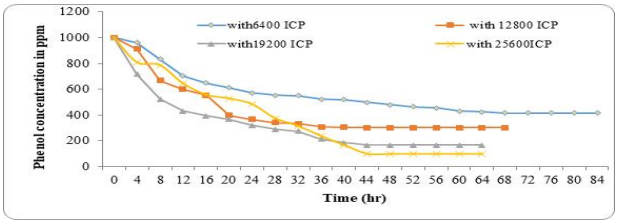

Fig. 4. Effect of quantity of immobilized carrier particles used on effluent phenol concentration during Start up in a packed bed bioreactor with *Nocardiahydrocarbonoxydans* immobilized on glass beads

***Conditions**: Initial phenol concentration: 1000 ppm (a) Carrier particles size: 3mm (b) Constant influent flow rate: 400 ml/hr*

(b) Initial phenol concentration: 500 ppm

The decrease in effluent phenol concentration at 4 hr during start up phase from initial phenol concentration (500 ppm) to 482 ppm for 6400 carrier particles and it was 204 ppm for 12800 carrier particles (Figure 5). The lowest effluent phenol from packed bed bioreactor having 6400 and 12800 carrier particles of 98 ppm and 60.98 ppm was obtained at 72 hr and 52 hr, respectively. The time taken to attain steady levels of phenol for 12800 carrier particles was observed to be lesser than that for 6400 carrier particles, which indicates rapid rate of phenol degradation with elevated number of carrier particles in packed bed bioreactor.

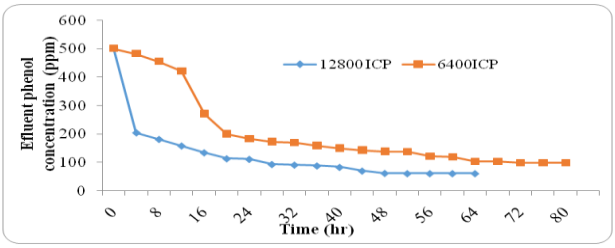

Fig. 5. Effect of quantity of immobilized carrier particles used on effluent phenol concentration during Start up in a packed bed bioreactor with *Nocardiahydrocarbonoxydans* immobilized on glass beads

***Conditions**: Initial phenol concentration: 500 ppm (a) Carrier particle size: 3 mm (b) Constant influent flow rate: 400 ml/hr*

Effect of quantity of immobilized carrier particles used on free cell concentration

(a) Initial phenol concentration: 1000 ppm

Figure 6shows the variation in free cell concentration during start up phase with the quantity of immobilized carrier particles in a packed bed bioreactor having *Nocardiahydrocarbonoxydans* immobilized on glass beads at initial phenol concentration of 1000 ppm. The increase in free cell concentration for 6400 carrier particles was 6.81 at 28 hr and for 25600 carrier particles it

was 181.36 at 56 hr during start up phase. The 12800 and 19200 carrier particles have shown the increase in free cell concentration from 1.36 to 24.06 and from 4.09 to 68.10 free cells, respectively. The decrease in free cells was observed after 28 hr, 32 hr, 40 hr and 56 hr for 6400, 12800, 19200 and 25600 carrier particles, respectively. The steady state free cell concentration for 6400-25600 carrier particles varied from 0.46 to 181.36 free cells where it was lowest for 6400 and highest for 25600 carrier particles. The results showed an increase in time to attain low and steady free cell concentration with increase in number of immobilized carrier particles in the packed bed bioreactor, which indicates high number of active cells sustained for longer duration with the increase in quantity of carrier particles.

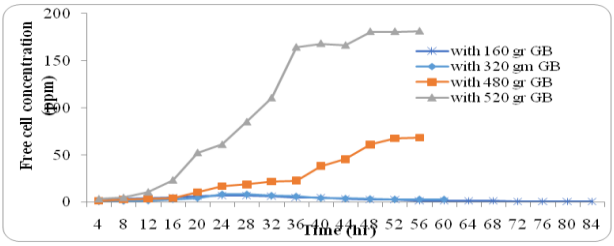

Fig. 6. Effect of quantity of immobilized carrier particles used on free cell concentration during start up in a packed bed bioreactor with *Nocardiahydrocarbonoxydans* immobilized on glass bead

Conditions: Initial phenol concentration: 1000 ppm (a) Carrier particles size: 3 mm(b) Constant influent flow rate: 400 ml/hr

(b) Initial phenol concentration: 500 ppm

Figure 7shows the free cell concentration at various quantities of immobilized carrierparticles during start up in a packed bed bioreactor with *Nocardiahydrocarbonoxydans* immobilized on glass beads at initial phenol concentration of500 ppm. The 6400 and 12800 carrier particles showed increase in free cell concentration from 1.36 to 39.54 and 7.27 to 23.18 free cells during start up phase up to 64 hr and 32 hr, respectively. The steady state free cell concentration for 6400 carrier particles (39.54) was found to be higher than that for 12800 carrier particles (7.72 at 52 hr).The results indicate high rate of detachment of cells from glass beads and shear rate for 6400 carrier particles than for 12800 carrier particles. Therefore the 12800 carrier particles placed in packed bed bioreactor would have high efficiency of phenol biodegradation.

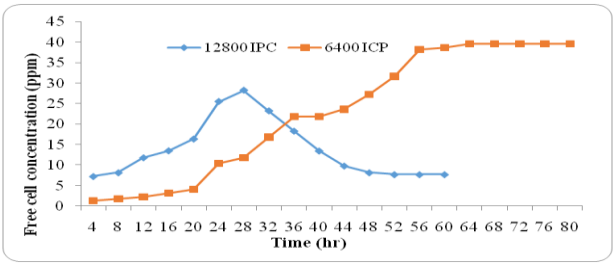

Fig. 7. Effect of quantity of immobilized carrier particles used on free cell concentration during start up in a packed bed bioreactor with *Nocardiahydrocarbonoxydans* immobilized on glass beads

Conditions: Initial phenol concentration: 500 ppm (b) Carrier particles size: 3 mm (c) Constant influent flow rate: 400 ml/hr

Conclusion:

The degradation of synthetic phenol waste water using immobilized glass beads with different biomass like *NocardiaHydrocarbonoxydans, Saccharomyce Cerevisiae* and *Candida Slooffi* were elaborately studied and it was observed that the growth rate of *Saccharomyce Cerevisiae* and *Candida Slooffi*was much more higher than*NocardiaHydrocarbonoxydans*indicating the former microorganism to be more robust in degrading phenol. The experimental studies revealed that the three microorganisms are useful in the biodegradation of phenol at a lower inlet concentration of upto 300 ppm. Packed bed was found to be cost effective for the purpose of degradation. It could be also observed that the inhibition effect is fess for *Candida Slooffi* and *NocardiaHydrocarbonoxydansand* hence can be useful for high concentration treatment. The effect of influent phenol concentration, the quantity of immobilized carrier particles and the dilution rate on effluent phenol concentration and on free cell concentration was elaborately studied for all the three types of microbes.

References

1. Chapman, Deborah V., ed. "Water quality assessments: a guide to the use of biota, sediments and water in environmental monitoring." (1996).
2. Ashton, Peter J., et al. "An overview of the impact of mining and mineral processing operations on water resources and water quality in the Zambezi, Limpopo and Olifants Catchments in Southern Africa, University of Zimbabwe-Harare. Report No. ENV-PC 42 (2001).
3. Aggarwal, Savita, GeetaPunhani, and JagritiKher. "Hot Spots of Household Water Insecurity in India's Current and Future Climates: Association with Gender Inequalities." Journal of Gender and Water 3.1 (2014): 34-41.
4. Tamburlini, Giorgio, Ondine Solveig von Ehrenstein, and Roberto Bertollini, eds. Children's health and environment: A review of evidence. Copenhagen: European Environment Agency, 2002.
5. El-Shahawi, M. S., et al. "An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants." Talanta 80.5 (2010): 1587-1597.
6. Kennish, Michael J. "Environmental threats and environmental future of estuaries." Environmental conservation 29.01 (2002): 78-107.
7. Berke, Philip R., and Maria Manta Conroy. "Are we planning for sustainable development? An evaluation of 30 comprehensive plans." Journal of the American planning association 66.1 (2000): 21-33.
8. Bernal, Blanca, and William J. Mitsch. "Comparing carbon sequestration in temperate freshwater wetland communities." Global Change Biology 18.5 (2012): 1636-1647.
9. Bandhyopadhyay.K ,Das.D , Bhattacharyya.P, Maiti.B.R (2001) Reaction Engineering studies on biodegradation of phenol by pseudomonas putida MTCC 1194 immobilized on calcium alginate, Biochemical Engineering Journal, Volume 8, Issue 3, October2001,Pages179-186.
10. Davis, J. B., and RoL Raymond. "Oxidation of alkyl-substituted cyclic hydrocarbons by a Nocardia during growth on n-alkanes." *Applied microbiology* 9.5 (1961): 383-388.
11. Erşan, Yusuf Çağatay, Nele De Belie, and Nico Boon. "Microbially induced CaCO 3 precipitation through denitrification: an optimization study in minimal nutrient environment." *Biochemical Engineering Journal* 101 (2015): 108-118.
12. Brown, Derick G., Joseph R. Stencel, and Peter R. Jaffé. "Effects of porous media preparation on bacteria transport through laboratory columns." *Water research* 36.1 (2002): 105-114.
13. Hahn, Martin W., et al. "The filtration–acclimatization method for isolation of an important fraction of the not readily cultivable bacteria." Journal of microbiological methods 57.3 (2004): 379-390.
14. Kumar, Arinjay, Shashi Kumar, and Surendra Kumar."Biodegradation kinetics of phenol and catechol using Pseudomonas putida MTCC 1194."Biochemical Engineering Journal 22.2 (2005): 151-159.
15. Shetty, K. Vidya, I. Kalifathulla, and G. Srinikethan."Performance of pulsed plate bioreactor for biodegradation of phenol." Journal of Hazardous Materials140.1 (2007): 346-352.
16. Pekguzel, E. A., T. K. Gundogdu, and N. Azbar. "Enhancement of Biohydrogen Production via Thermophilic Cell Culture Immobilized on Glass Beads and Raschig Rings of Different Sizes in a Packed Bed Reactor."Chemical and Biochemical Engineering Quarterly 29.4 (2016): 541-547.
17. Venter, J. Craig, et al. "Biologically active catecholamines covalently bound to glass beads." Proceedings of the National Academy of Sciences 69.5 (1972): 1141-1145.
18. D'souza, S. F., et al. "Immobilization of yeast cells by adhesion to glass surface using polyethylenimine." Biotechnology letters 8.9 (1986): 643-648.