Aerobic biodegradation of phenol by Immobilized *Pseudomonas sp.* cells in two different bio-carrier matrices

Prof. Zainab Ziad Ismail
Department of Environmental Engineering
College of Engineering
Baghdad University
Email: zismail9@gmail.com

Asst. Lect. Haneen Ahmed Khudhair
Department of Environmental Engineering
College of Engineering
Tikrit University
Email: ayacherry_2008@yahoo.com

ABSTRACT

Biotreatment using immobilized cells (IC) technology has proved to be the most promising and most economical approach for the removal of many toxic organic pollutants found in petroleum-refinery wastewater (PRW) such as phenol. This study was undertaken to evaluate the degradation of phenol by *Pseudomonas* cells individually immobilized in two different bio-carrier matrices including polyvinyl alcohol-guar gum (PVA-GG) and polyvinyl alcohol-agar agar (PVA-AA). Results of batch experiments revealed that complete removal of phenol was attained in the first cycle after 150 min using immobilized cells (IC) in both PVA-GG and PVA-AA. Additional cycles were confirmed to evaluate the validity of recycling beads of immobilized cells for phenol biodegradation. Results revealed that the phenol percentage removals were 95, 92, 86, and 84 % for second, third, fourth, and fifth cycles, respectively using *Pseudomonas* immobilized in PVA-GG beads. Whereby they were 96, 92, 90, and 84 % using *Pseudomonas* immobilized in PVA-AA beads for the same sequence of cycles.

Key words: Immobilized cells, *Pseudomonas*, bio-carrier, wastewater, and phenol

التحلل الهوائي للفينول باستخدام خلايا البكتريا الزائفة المقيدة في نوعين من الحوامل الاحيائية

أ. زينب زياد اسماعيل
قسم الهندسة البيئية
كلية الهندسة / جامعة بغداد

م.م. حنين أحمد خضير
قسم هندسة البيئة
كلية الهندسة / جامعة تكريت

الخلاصة

ان المعالجة الاحيائية باستخدام تقنية الخلايا المقيدة من اهم الاساليب الحديثة والمجدية اقتصاديا لازالة معظم المواد العضوية المثبطة والمقاومة للتحلل البايولوجي والتي تتواجد ضمن مخلفات المصافي النفطية ومن ضمنها الفينول. اذ تم اجراء هذا البحث لغرض دراسة تحلل الفينول بتركيز ابتدائي 20 ملغم/لتر باستخدام خلايا البكتريا الزائفة والمقيدة ضمن نوعين من الحوامل الاحيائية وهما البولي فينيل الكحول- صمغ البازلاء والبولي فينيل الكحول- المادة الطحلبية الهلامية. بينت النتائج التجريبية عملية ازالة كلية للفينول بعد 150 دقيقة في الدورة الاولى باستخدام الخلايا المقيدة. تم استخدام خرز الخلايا المقيدة في دورات اعادة استخدام اضافية لتقييم اعادة استخدام تلك الخرز في عملية التحلل البايولوجي للفينول. وقد كانت النسب المئوية لازالة الفينول كالتالي: 95، 92، 86 و 84 % للدورات الثانية، الثالثة، الرابعة والخامسة على التوالي بعد مرور 150 دقيقة باستخدام الخلايا المقيدة في خرز PVA-GG، في حين كانت نسب الازالة: 96، 92، 90 و 84 % لنفس تسلسل الدورات على التوالي باستخدام الخلايا المقيدة في خرز PVA-AA.

الكلمات الرئيسيه: الخلايا المقيدة، *Pseudomonas*، الحامل الاحيائي ، مياه الصرف الصحي، والفينول .

1. INTRODUCTION

Phenol is the first compound inscribed into the list of priority pollutants by the US Environmental Protection Agency (US EPA). Phenol irritates skin and causes its necrosis; it damages kidneys, liver, muscle and eyes, Phenolic compounds are carcinogenic to human and lethal to aquatic life at relatively low concentration levels (5-25) mg/L, **Michalowicz, and Duda, 2007; Al-Khalid, and El-Naas, 2012**. The maximum allowable concentration of phenol is 0.5 mg/L according to the Iraqi legislation. Thus; the removal of phenol from industrial aqueous effluents is of great practical significance for environmental protection, **Al Zarooni, and Elshorbagy, 2006; El-Naas et al., 2010**. The physical and chemical treatment methods have limited success when applied to the treatment of refractory organic pollutants because these processes led to secondary effluent problems due to formation of toxic materials. Therefore, intensive attention has been paid to the degradation of these pollutants by microorganisms and their transformation into compounds inoffensively with formation of new cellular mass, **Karigar, et al., 2006; Cozma, et al., 2012**. Biotreatment of phenol has attracted great attention due to its environmentally friendly approach and its ability to mineralize toxic organic compounds completely. However, phenol degradation by bacterial cells generally limited by substrate inhibition and low conversion rates. These drawbacks can be overcome by immobilization technology. Immobilized cells (IC) technology utilizes the colonization of specialized bacterial cells into beads made from natural and synthetic polymers. Bacteria of the genus *Pseudomonas* are a group of increasing both fundamental and biotechnological interest that exhibit a diverse range of metabolic activities and play an important role in the degradation of aromatic hydrocarbon in the PRW, **Kuyukina et al, 2009**. However, toxicity of organic pollutants may prevent or slow metabolic reactions in biological treatment. So, the introduction of new and improved biotechnologies that enable engineers and scientists to tackle the more contemporary environmental problems such as detoxification of hazardous compounds through the use of living microorganisms would be necessity, **Loh, et al., 2000; Martins, et al., 2013**. It has been shown that the biodegradation rate can be improved by immobilizing the cells by using solid support particles such as polyvinyl alcohol (PVA) to obtain the maximum degradation capability, **Al-Khalid, and El-Naas, 2012**. There were a little previous works that dealt with relatively low initial phenol concentration. However, **El-Naas, et al., 2009** evaluated phenol biodegradation by *Pseudomonas Putida* immobilized in PVA gel pellets at initial phenol concentration of 75 mg/L. In the study done by **Chung, et al., 2003** that compared between free and immobilized cells of *Pseudomonas putida*, free cells (FC) could degrade phenol up to about 600 mg/L in batch reactor whereas this level becomes up to 1000 mg/L for immobilized cells (IC) in Ca-alginate beads. **Wang, et al., 2007** used PVA carrier for immobilization and reported that the immobilized cells of *Acinetobacter sp.* could tolerate higher phenol levels and the immobilized cells possessed better storage stability, which demonstrated that PVA carrier cubes had good flexibility and retained a high mechanical strength. **Zhiguo, et al., 2012** isolated a bacterial strain *Pseudomonas sp*. that was capable of degrading nitrobenzene, phenol, aniline, and other aromatics and then immobilized its cells in the mixed carrier of polyvinyl alcohol and sodium alginate to improve its degrading efficiency. In the study of **El-Gendy, and Nassar, 2015** was isolated the marine diesel-oil degrading bacterium, *Pseudomonas aeruginosa* NH1 and examined its ability to degrade diesel oil contaminating seawater as immobilized cells by entrapment in Ca-alginate. The biodegradation rate of different components of diesel oil was enhanced by immobilization, indicating the improved

tolerance of the immobilized cells towards different toxic components of diesel oil and environmental conditions.

The aim of this study is to evaluate the phenol biodegradation by immobilized cells of the bacterium of genus *Pseudomonas*. Two natural bio-carrier polymers; guar gum (GG) and agar-agar (AA) reinforced by Polyvinyl alcohol (PVA), were used to immobilize the bacterium of genus *Pseudomonas* for investigating the performance of the suggested system in enhancement phenol removal efficiency.

2. MATERIALS AND METHODS

The materials which were used as bio-carriers in this study were natural polysaccharide polymers including Agar-Agar (AA) and Guar Gum (GG) as given in **Table 1**. These biopolymers were individually cross-linked with polyvinyl alcohol (PVA) to improve the strength and mechanical stability of beads since the natural polysaccharides are abundantly available but they are less stable in the wastewater than synthetic polymers, **Stolarzewicz, et al., 2011**.

2.1 Isolation of inoculum for use in the preparation of immobilized cells

A phenol-degrading bacterial strain was isolated from the activated sludge freshly collected from Baiji Oil Refinery, (Iraq), and identified as *Pseudomonas* sp. via enrichment culture technique which included streaking the bacterial strain on Petri dishes. Three-3-day-old colonies that incubated at 25 °C on Inoculum Production Medium (IPM) agar were transferred into 5 ml of IPM and incubated statically for 48 h at 25 °C. Subsequently, this culture was transferred into 250 ml screw-capped Erlenmeyer flask containing 45 ml of IPM. The loosely capped flask was incubated for 24 h in an orbital shaker at 25 °C and 150 rpm, **Safont, et al., 2012**.

2.2 Immobilization Protocol

2.2.1 Polyvinyl alcohol-guar gum matrix

PVA-GG matrix was prepared as follows: 6 g PVA and 2 g GG were dissolved in 100 ml distilled water and blended by a magnetic stirrer at 70 °C for 30 min. After cooling the mixture to room temperature, it was inoculated with 5 ml of inoculum culture prepared from previous step and was stirred by a magnetic stirrer for 15 min. The obtained solution was poured into sterile micro-plates to form beads of 3 mm diameter contained immobilized cells, kept in the freezer for 12 hours, and then thawing them. The freezing-thawing procedure was repeated for 3 times to improve the stability of beads, **Bai, et al., 2010**.

2.2.2 Polyvinyl alcohol-agar agar matrix

A 2% solution of Agar-Agar was prepared with 0.9 % PVA powder. A 5ml volume of biomass inoculum was added to the PVA-agar mixture, shaken well for few seconds (without

forming entrapped bubbles), poured into 2 sterile micro-plates and allowed to solidity in the freezer and then thawed. The beads were washed with distilled water 3 to 4 times, **Kumer, et al., 2014**. **Fig. 1** presents samples of the prepared beads with 3 mm in diameter.

2.3 Culture Medium

1 L of the mineral salt medium (MSM) for inoculum cultivation consisted of (g L^{-1}): NaCl, 1; Na_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.2; and yeast extract, 0.5, in distilled water (DW). The pH of MSM was adjusted to 7. To prepare the culture medium containing carbon and energy sources, an appropriate amount of phenol from a stock solution of 20 mg/L (stored in a dark bottle at 4°C) was firstly dispended into 250 ml flasks. MS medium was then added to the 500 ml flasks and finally the resulted aqueous solution was added to spouted bed bioreactor (SBBR).

2.4 Experimental System Configuration and Setup

The experimental system consisted of a specially designed fluidized bed bioreactor, known as spouted bed bioreactor (SBBR) made of Perspex column (inner diameter 50 mm, height 70 cm) with 45° conical base. The SBBR could be operated in batch and continuous mode operation as well. The SBBR was outfitted with a Perspex jacket (inner diameter 80 mm) for temperature control. A water bath was designed to continuously circulate the water at a desired temperature of 30°C. The water bath consisted of 6 Liter-cylindrical Perspex tank, occupied with heater (Aquarium immiscible heater rod, China) and water pump (RS Electrical, RS-80, Q_{max} = 1000 L/h & H_{max} = 1m) to circulate the water into the reactor jacket. The aqueous solutions were fed to the SBBR via a peristaltic pump (Thomas 3386). In order to provide an intense mixing and maintain aerobic environment into the spouted bed bioreactor, air was injected from the bottom of the reactor by an air pump (HAILEA, ACO-308, China) through a 6 mm-orifice. A flow meter and controller (GENTEK, GNT604) was provided to control the air flow into the system. The experimental setup is given in **Fig. 2**. The spouted bed bioreactor reactor was operated in an up flow co-current air/water mode with 4 L/min and 20 ml/min for air and liquid flowrates, respectively.

2.5 Analytical Analysis and Methodologies

Phenol concentration in aqueous samples was determined by T80 UV-VIS Spectrophotometer at 270 nm wavelength. The cells counting in each experiment with immobilized microorganisms involves removal of the cells from the immobilization matrix by dissolving the beads by immersing them in 4 % $NaHCO_3$ solution for 30 min. Samples of microorganisms residing in the wastewater were used without additional treatment. Routine counts of biomass cells in wastewater or within the beads were counted by the Plate Count Method (CFU/mL) in a series of dilutions (in 0.85 % saline) and traditional approach of volatile suspended solids (VSS, g/L), **Cruz, et al., 2013**.

3. RESULTS AND DISCUSION

3.1 Effect of Beads Concentration on Biodegradation Rate

The amount of immobilized cells beads in the bioreactor plays an important role in the biodegradation process. The performance of immobilized cells was examined at initial concentration of 500 mg/L equivalent to free cells system by using 10 and 25 % (v/v) of beads. In order to make a reasonable comparison between biodegradation rates at the two bead densities (10 and 25 % (v/v)) examined in this study, the initial bacterial cells concentration was kept in the beads and increasing the number of beads at lower cell density inside them. This can be explained by the fact that the higher cell concentration inside the beads limits the nutrients availability inside beads. This could have in turn contributed to the enhancement and improvement of biodegradation process, **Safont, et al., 2012**. **Fig. 3** shows the effect of beads concentration (as volume percentage) on the biodegradation of phenol. The degradation rates of phenol using 10 % (v/v) of immobilized cells in PVA-GG and PVA-AA beads were 5.298 and 6.240 mg/L.h, respectively. Whereby, they were 7.50 and 7.53 mg/L.h, respectively using 25 % (v/v). Hence, the biodegradation rate using 25 % (v/v) of beads was higher than the biodegradation rate using 10 % (v/v). It is well expected since the concentration of the beads in the bioreactor can be related directly to the amount of the bacterial biomass immobilized in these beads. The results which performed by **Loh, et al., 2000,** who used an immobilized-cell membrane bioreactor to investigate phenol degradation at high concentrations using Pseudomonas Putida, showed that 25 % (v/v) of immobilized cells were effective in rapid degradation of 100 mg phenol/L.

3.2 Time Course of Phenol Biodegradation

Time course of phenol biodegradation was examined at initial phenol concentration of 20 mg/L by taking a sample periodically every 30 min. Results given in **Fig. 4** and **Fig. 5** showed that the bacterium of *Pseudomonas sp.* was capable to complete removal of 20 mg/L of phenol after 150 min for the bacterial cells immobilized in both PVA-GG and PVA-AA beads at the 1^{st} degradation cycle without any further acclimation to the phenol initial concentration. These results were compared to results that performed by **El-Naas, et al., 2009,** which indicated that the bacterium of *Pseudomonas Putida* was capable to complete removal of 25 mg/L of phenol after 48 min for the bacterial cells immobilized in PVA gel pellets due to previous acclimation process to the phenol which the microorganism used as a source of carbon and energy. According to **Karigar, et al., 2006**, there was a decline in the growth rate of immobilized cell of *Arthrobacter citreus* due to the inhibitory effects of phenol at 22mM.

3.3 The Potential of Using Immobilized Cells for Phenol Degradation

Results demonstrated complete degradation of phenol can be obtained using Pseudomonas immobilized in PVA-GG and PVA-AA beads in the first cycle after 150 min. In addition, to determine the validity of recycling immobilized cells of *Pseudomonas* was determined by carrying out consecutively excessive batch experiments using recycled beads. Results revealed that immobilized cells can be efficiently reused without decline in their biodegradation capability. The results of the phenol percentage removals for the last four excessive cycles were as following: 95, 92, 86, and 84 % for second, third, fourth, and fifth cycles, respectively using Pseudomonas immobilized in PVA-GG beads at degradation

period of 150 min. Whereby they were 96, 92, 90, and 84 % using Pseudomonas immobilized in PVA-AA beads for the same sequence of cycles. According to the results noted by, **Wang, et al., 2007,** the immobilized cells of *Acinetobacter sp*. degraded 69% of 100 mg phenol/L after two reuse cycles.

In this study as shown in **Fig. 4** and **Fig. 5**, this phenomenon could reduce expenses during operational periods, **Cai, et al., 2011**. However, the biodegradation rates of phenol were decreased slightly and gradually with the consecutive cycles due to the mechanical instability of beads and gradual leakage from their porous.

4. CONCLUSION

The physical freezing-thawing method of immobilization proposed in this study was shown to be a successful with natural polymers in phenol biodegradation. The biodegradation rate of phenol using *Pseudomonas* cells individually immobilized in PVA-GG and PVA-AA matrices was evaluated in a spouted bed bioreactor (SBBR). Experimental results demonstrated that complete removal of phenol can be obtained after 150 min using immobilized cells in both PVA-GG and PVA-AA beads. Also, the use of immobilized cells (IC) allowed to increase the number of biodegradation process cycles, but relatively reduced the degradation rate. However, excessive degradation cycles up to 5 cycles were carried out and results revealed that the potential recycling of immobilized cells for complete removal of phenol at different time periods. Results indicated that increasing the beads concentration resulted in the increasing the biodegradation rate of phenol since the increasing of beads resulted in increasing the biocatalyst cells concentration in these beads and therefore the degradation rate would be higher.

REFRENCES

- AL-Khalid, T., and EL-Nass, M., 2012, *Aerobic Biodegradation of Phenols: A Comprehensive Review. Critical Reviews,* Journal of Environmental Science and Technology, Vol.42, PP. 1631–1690.

- Al Zarooni, M., and Elshorbagy, W., 2006, *Characteristics and Assessment of Al Ruwais Refinery Wastewater,* Journal of Hazardous Materials, Vol.136, No.3, PP. 398-405.

- Bai, X., Ye, Z., Li, Y., Yang, L., Qua, Y., and Yang, X., 2010, *Preparation and Characterization of a Novel Macroporous Immobilized Microorganism Carrier,* Journal of Biochemical Engineering, Vol.49, PP. 264–270.

- Cai, T., Chen, L., Ren, Q., Cai, S., and Zhang, J., 2011, *The Biodegradation Pathway of Triethylamine and Its Biodegradation by Immobilized Arthrobacter protophormiae Cells,* Journal of Hazardous Materials, Vol.186, PP. 59-66.

- Chung, T.P., Tseng, H.Y., and Juang, R.S., 2003, *Mass transfer effect and intermediate detection for phenol degradation in immobilized Pseudomonas putida Systems*, Vol.38, No.10, PP. 1494-1507.

- Cozma, P., Hlihor, R., Apostol, L.C., Diaconu, M, Pogancean, M.O., and Gavrilescu, M., 2012, *Aerobic Biodegradation of Phenol by Activated Sludge in a Batch Reactor,* Journal of Environmental Engineering and Management, Vol.11, PP. 2053-2058.

- Cruz, I., Bashan, Y., Carmona, G., and Bashan, L., 2013, *Biological Deterioration of Alginate Beads Containing Immobilized Microalgae and Bacteria During Tertiary Wastewater Treatment,* Journal of Applied Microbiological Biotechnology, Vol.97, PP. 9847-9858.

- El-Gendy, N.Sh., and Nassar, H.N., 2015, *Kinetic Modeling of The Bioremediation of Diesel Oil Polluted Seawater Using Pseudomonas aeruginosa NH1*., Journal of Energy Sources, part A: Recovery, Utilization, and Environmental Effects, Vol.37, PP.1147-1163.

- El-Naas, M.H., Al-Muhtaseb, S.A., and Makhlouf, S., 2009, *Biodegradation of Phenol by Pseudomonas Putida Immobilized in Polyvinyl Alcohol (PVA) Gel*, Journal of Hazardous Materials, Vol.164, PP. 720-725.

- El-Naas, M., Al-Zuhair, S., and Makhlouf, S., 2010, *Batch Degradation of Phenol in a Spouted Bed Bioreactor System,* Journal of Industrial and Engineering Chemistry, Vol.16, PP. 267–272.

- Karigar, C., Mahesh, A., Nagenahalli, M., and Yun, D.J., 2006, *Phenol Degradation by Immobilized Cells of Arthrobacter citreus,* Journal of Biodegradation, Vol.17, PP. 47–55.

- Kumar, D., Chauhan, P., Puri, N., and Gupta, N., 2014, *Production of Alkalin Thermostable Protease by Immobilized Cells of Alkalophilic Bacillus sp. NB 34*, International Journal of Current Microbiology & Applied Science, Vol.3, No.10, PP. 1063-1080.

- Kuyukina, M.S., Ivshina, I.B., Serebrennikova, M.K., Krivorutchko, A.B, Podorozhko, E.A., Ivanov, R.V., and Lozinsky, V.I., 2009, *Petroleum-Contaminated Water Treatment in Fluidized-Bed Bioreactor with Immobilized Rhosococcus Cells,* Journal of International Biodeterioration and Biodegradation, Vol.63, PP. 427-432.

- Loh, K.C., Chung, T.S., and Ang, W.F., 2000, *Immobilized-Cell Membrane Bioreactor for High Strength Phenol Wastewater,* Journal of Environmental engineering, January, PP. 75-79.

- Martins, S.C., Martins, C.M., and Fiuza, L.M., 2013, *Immobilization of microbial cells: a promising tool for treatment of toxic pollutants in industrial wastewater*, African Journal of Biotechnology, Vol.12, No.28, PP. 4412-4418.

- Michalowicz, J., and Duda, W., 2007, *Phenols – Sources and Toxicity*, Journal of Environmental Studies, Vol.16, No.3, PP. 347-362.

- Safont, B., Vitas, A.I., and Peñas, F.J., 2012, *Isolation and Characterization of Phenol Degrading Bacteria Immobilized onto Cyclodextrin-Hydrogel Particles within a Draft Tube Spouted Bed Bioreactor*, Journal of Biochemical Engineering, Vol.64, PP. 69-75.

- Stolarzewicz, I., Bialecka-Florjañczyk, E., Majewska, E., and Krzyczkowska, J., 2011, *Immobilization of Yeast on Polymeric Supports*, Journal of Chemical Biochemistry Engineering, Vol.25, PP. 135-144.

- Wang, Y., Tian, Y., Han, B., Zhaw, H., Bi, J., and Cai, B., 2007, *Biodegradation of Phenol by Free and Immobilized Acinetobacter sp. Strain PD12*, Journal of Environmental Science, Vol.19, PP. 222–225.

- Zhiguo, W., Lin, Y., Liu, H., Yia, Y., Shen, W., Hong, Q., Li, S., and Yao, H., 2012, *Characterization of The Nitrobenzene-degrading Strain Pseudomonas sp. a3 and use of its immobilized cells in the treatment of mixed aromatics wastewater*, Journal of Microbial Biotechnology, Vol.28, PP. 2679-2687.

NOMENCLATURE

AA: Agar-agar

CFU: Colony Forming-Unit

DW: Distilled water

FC: Free cells

GG: Guar gum

IC: Immobilized cells

IPM: Inoculum Production Medium

MSM: Mineral Salt Medium

PRW: Petroleum-Refinery Wastewater

PVA: Polyvinyl alcohol

rpm: revolution per minute

SBBR: Spouted bed bioreactor

VSS: Volatile Suspended Solids

Figure 1. Samples of the prepared beads.

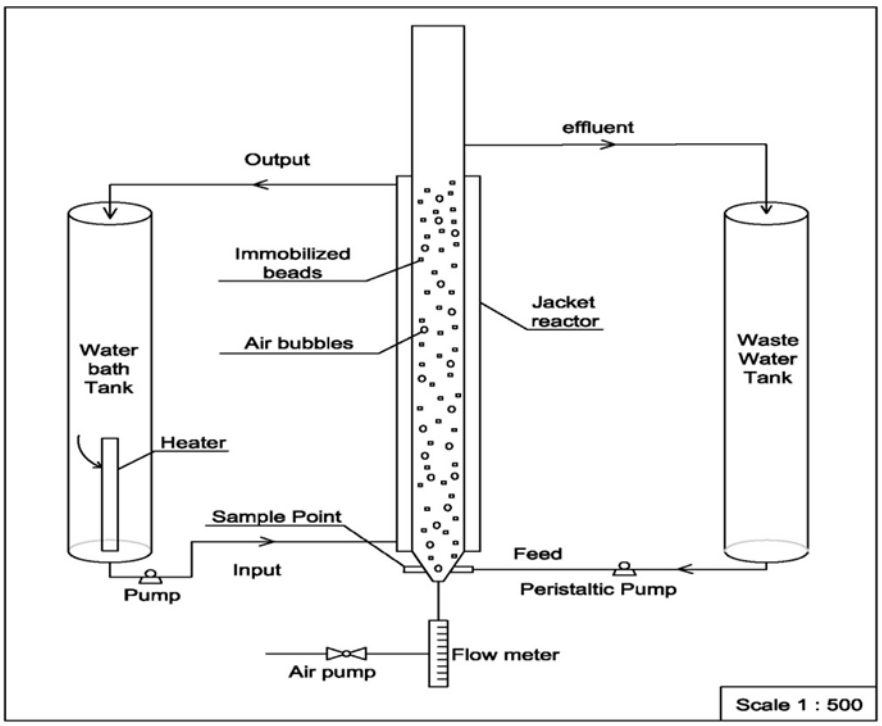

Figure 2. Schematic diagram of the lab-scale spouted bed bioreactor.

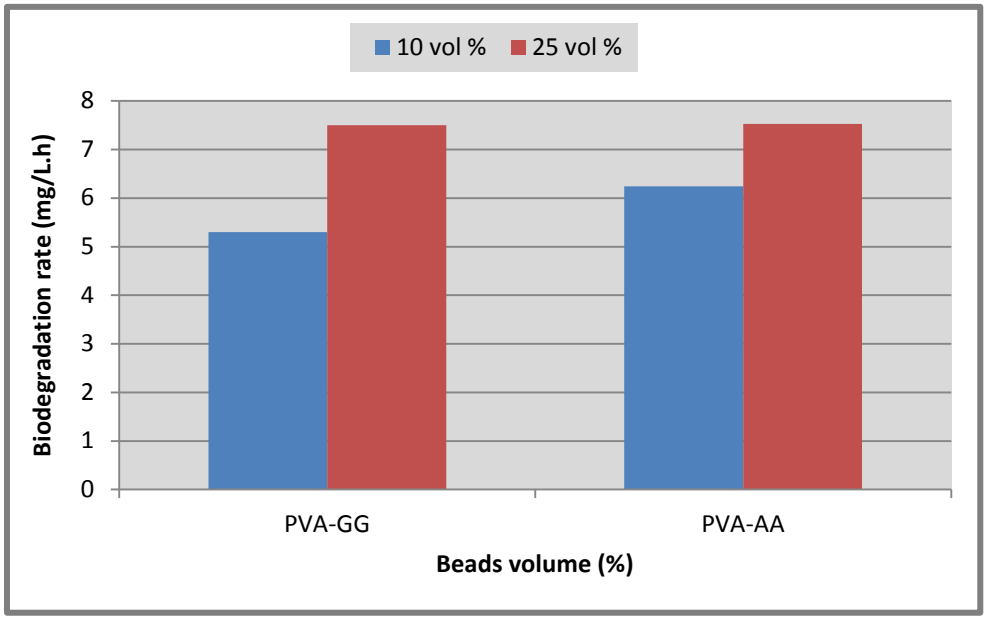

Figure 3. Biodegradation rate of phenol by Pseudomonas immobilized in two different bio-carriers using 20 mg/L initial phenol concentration .

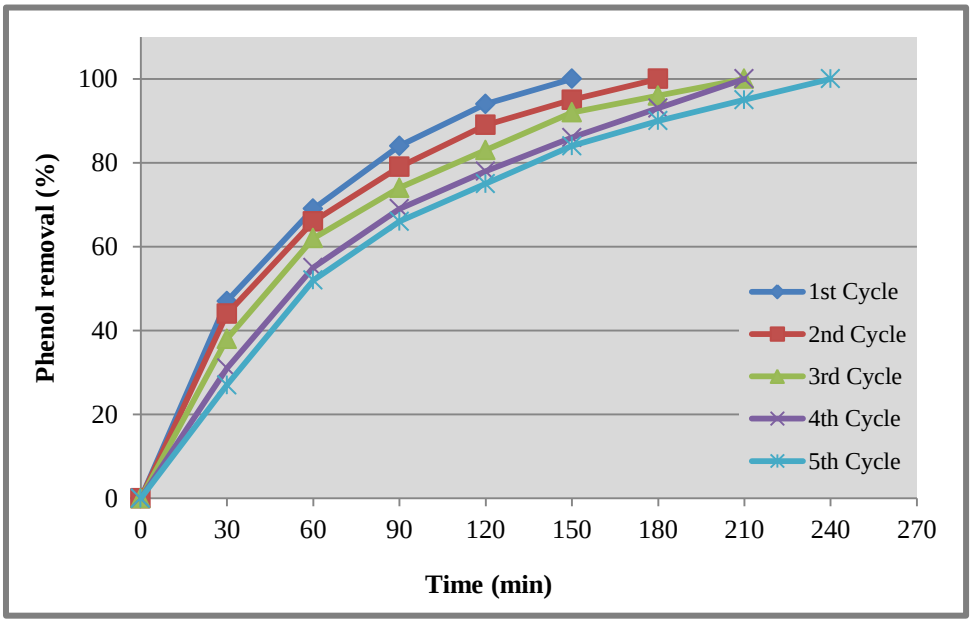

Figure 4. Profiles of phenol degradation by *Pseudomonas* cells immobilized in PVA-GG.

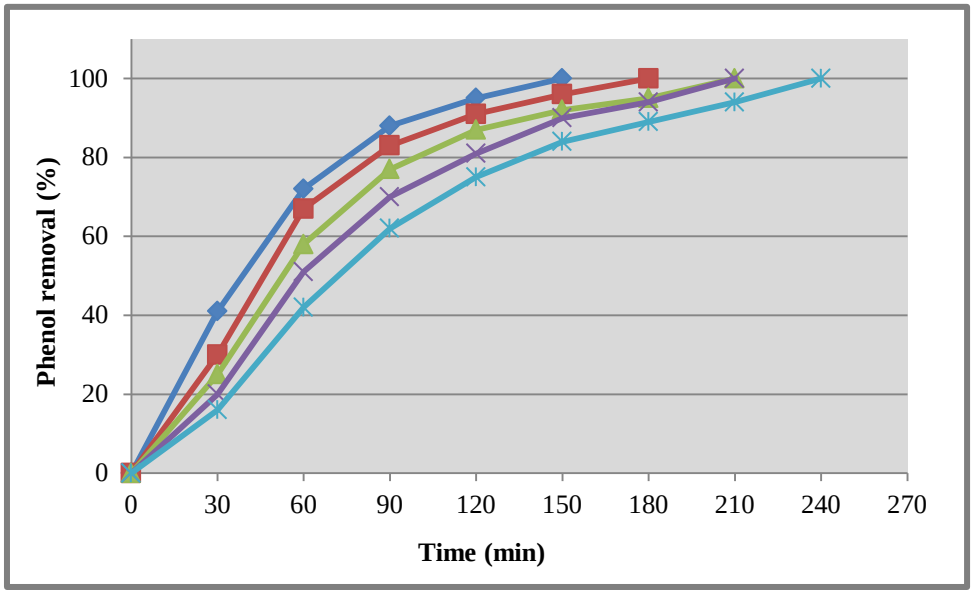

Figure 5. Profiles of phenol degradation by *Pseudomonas* cells immobilized in PVA-AA.

Table 1. Polymers used as bio-carriers in the study.

Name	Abbreviation	Chemical formula	Source	E. Code*
Polyvinyl alcohol	PVA	$(C_2H_4O)_n$	raw material of vinylon	———
Agar-Agar	AA	$(C_{12}H_{18}O_9)_n$	algal polysaccharides derivatives (Marine-Seaweed algae)	E406
Guar Gum	GG	$(C_6H_{10}O_5)_n$	Obtained from non-marine botanical resources	E412

*(E. Number): is a code for a substance that can be used as food additives within European Union & Switzerland.