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DEGREDATION OF PERCHLORATE WITH PHENOL AS A SOLE CARBON SOURCE BY AN ISOLATED STRAIN *PSEUDOXANTHOMONAS SP.*

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ABSTRACT: Phenol was used as a sole carbon source for the perchlorate biodegradation as reported for the first time. Isolated bacterial species *Pseudoxanthomonas* sp. isolated from a sewage sludge consortium was found to reduce perchlorate while taking phenol as electron acceptor. The growth and perchlorate degradation were analyzed in a batch system along with the degradation profile of phenol by the microbial culture. The effect of the co-pollutants (nitrate, chlorate, nitrate, phosphate) was also considered as part of the present study, where nitrate was found to be the most significant. Perchlorate was significantly removed by the culture involving subsequent phenol degradation in a continuous packed bed system anaerobically from synthetic wastewater.

INTRODUCTION: Perchlorate ion consists of a tetrahedral array of oxygen atoms around a central chlorine atom. As the oxidation state of the chlorine is +7, the species is a strong oxidizing agent. However, perchlorate reduction is extremely nonlabile (slow) and can usually be observed only in concentrated strong acid. In fact, the redox behavior of perchlorate is so rarely observed in chemical systems that sodium perchlorate is used to adjust the ionic strength of solutions before electrochemical or other laboratory studies [1]. Complexes of perchlorate are usually rare and noteworthy when encountered.

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Perchlorate does not sorb well to most surfaces, and most perchlorate salts are quite soluble (upto 2 kg/l in water in the case of sodium perchlorate). Notable exceptions include tetraphenyl arsonium perchlorate and some quaternary ammonium perchlorate salts, which form stable ion pairs. Perchlorate salts share natural as well as anthropogenic sources. Perchlorate has been found in groundwaters in the United States, at typical concentrations of 50-200 mg/l, primarily as a result of production and uses in solid rocket propellant [2].

Most of the affected regions have perchlorate concentrations below 0.5 g/l; however, concentrations as high as 3.7 g/l have also been encountered. Perchlorate in sewage sludge, rice, bottled drinking water, and milk was detected in China to investigate perchlorate pollution status [3]. Perchlorate is known to interfere with the uptake of iodine in the thyroid at the (Na^+)-iodide (I^-) symporter, or NIS of the thyroid gland, thereby

causing a reduction in the hormones thyroxine (T_4) and tri-iodothyronine (T_3) [4]. Hyperthyroidism due to iodine deficiency during pregnancy is a cause of cretinism, a permanent cognitive impairment of the developing fetus. Perchlorate is thought to be responsible for abnormal fetal and child growth and development [1, 2, 5]. In some cases, thyroid gland tumors can be caused due to disruptions in thyroid hormone levels. The office of Environmental Health Hazard Assessment in California EPA has proposed a Public Health Goal of 6µg/l for perchlorate in drinking water. In the National Academy of Science's (NAS's) January 2005 report, the maximum permissible dose for perchlorate was proposed to be 0.7 µg/kg/d [6]. Perchlorate treatment technologies can be divided into two primary categories, destruction, and removal. Removal technologies encompass broadly the physical and biological treatment.

Among physical methods electrochemical reduction, ion-exchange, membrane filtration, electrodialysis, catalytic reduction and biological treatment (phytoremediation and microbiological treatment processes). A recent report by U.S EPA indicates that ion exchange and bioremediation are among the most commonly used technologies to remove or degrade perchlorate from contaminated media. All the physicochemical technologies have certain disadvantages, for which bioremediation has been chosen as the suitable technique in many cases. High capital and maintenance cost, generation of a large quantity of brines, and spent resin having high perchlorate concentration.

Membrane fouling by alkaline earth and transitional metal compounds can present a problem depending on their concentration in the water. Biodegradation of perchlorate in engineered systems offers the greatest potential for inexpensive and complete perchlorate degradation. Several reactor technologies have been shown to treat perchlorate, for which three patents have been obtained [7]. Additionally, certain microbiota can cause irreversibly foul or damage the membrane material, necessitating complete replacement. The greatest challenge, in this case, will be to sustain enough biomass to create continuously reducing conditions without the addition of an extra amount of electron acceptors and donors. Combination of treatment technologies may be applicable under certain conditions. For example, a bioreactor could be used to reduced very high concentrations of perchlorate and other co-contaminants (*e.g.*, nitrate, chlorate, sulfate) followed by ion-exchange as a polishing step. According to Urbansky [1] bioremediation of perchlorate-contaminated water is promising. Perchlorate respiring bacteria (PRB) have been found many different environments making it possible to bioremediate perchlorate-contaminated environments [8, 9, 10, 11]. Perchlorate is used as a terminal electron acceptor by pure and mixed cultures of microorganisms [12]. The proposed perchlorate reduction pathway is [13]

$$ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl_2^- + O_2$$

The reduction of perchlorate or chlorate to chloride by bacteria was also subsequently confirmed by other researchers [11, 14, 15]. None of the intermediates accumulate in solutions [8, 12]. Chlorite disproportionation to chloride and oxygen is a non-energy yielding step catalyzed by chlorite dismutase [11, 15.]

Phenol has been used as the sole C-source for denitrification by some microbial cultures [16, 17, 18, 19, 20]. These nitrate reducers can accept phenol and other known aromatic hydrocarbons for their metabolism. The perchlorate reducers share many similar metabolic features with the denitrifiers, including their choice of electron donors (C-sources). Till date, there is no report of perchlorate reducers accepting phenol or any other aromatic hydrocarbons while degrading perchlorate. This is the first report of any PRB (perchlorate reducing bacteria) degrading both phenol and perchlorate simultaneously in water system. This unique property of the microbial consortium, predominantly *Pseudoxanthomonas* sp. can be very useful in the biological treatment system treating wastewater coming from different industries or contaminated sites having both of the pollutants, perchlorate, and phenol. In the past 20 years, there have been many reports on the biodegradation of phenol under anaerobic conditions. It has been shown that phenol is utilized by sulphate-reducing bacteria microorganisms participating in methanogenic fermentation (Wang *et al.,* 1986) and denitrifying bacteria [16]. Phenol is difficult to be decomposed biologically. It is toxic to plants, microorganisms, animals and humans, causing

serious environmental problems [21]. In addition, it is water-soluble and highly mobile, so it is likely to reach drinking water sources downstream from discharges, where, even at low concentrations, it can cause severe odor and taste problems and pose risks to populations. Phenolic compounds are present in the liquid effluent of coal gasification plants, coking plants, petroleum refineries, pharmaceutical, fertilizer and dye manufacturing plants, as well as degreasing and paint stripping operations [22] and fiber-board manufacturing [23] as a result of growing awareness over pollution caused by phenol release, efforts are being made to minimize their adverse effect.

Many treatment techniques such as activated carbon adsorption, solvent extraction, chemical oxidation, electrochemical oxidation, and biodegradation have been developed to remove phenol from contaminated environments [24] of these options, physicochemical methods have proven to be costly and have the inherent drawbacks of causing secondary pollution. However, the biodegradation technique, which is environmentally friendly and cost-effective, has turned out to be a favorable alternative [25]. Although not found to be, humans exposed to phenol in well water at concentrations of 1300mg l^{-1} exhibited a statistically significant increase in diarrhea, mouth sores, dark urine, and burning of the mouth [26.] The process has been carried out for many years, either aerobically or anaerobically.

Between the two, the latter is preferred because it saves the energy needed for aeration and produces a substantially lower amount of sludge. Anaerobic treatment of phenol-containing wastewater in upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB)-based bioreactors has been well documented. Two possible anaerobic degradation pathways of phenol under mesophilic conditions have been reported. In one suggested pathway [27], phenol is first converted through carboxylation to produce benzoate. The latter is then de-aromatized to form cyclohexanecarboxylate, which is further cleaved to form heptanoate. Heptanoate is then degraded either through b-oxidization to form valerate, propionate, and acetate [28], or directly to form propionate and butyrate, both of which can be further degraded into acetate [29]. This pathway was supported by enzymes performing carboxylation, decarboxylation, and dehydroxylation reactions during phenol anaerobic degradation [30]. In the other degradation pathway [31], phenol is reduced in the presence of nitrate to cyclohexanone and then n-caproate, which is subsequently undergone β-oxidation to form lower volatile fatty acids (VFAs).

MATERIAL AND METHODS:

Growth Medium and Conditions: Chemicals and reagents used in the study were of analytical grade, and inorganic salts used in preparing microbial growth media were of reagent grade. Sodium perchlorate ($NaClO_4$. H_2O), procured from Merck, India. Was used as the source of ClO_4^- in all the experiments. All the other chemicals used in this study were purchased from Merck, India. All the enrichments and growth of mixed cultures were performed in an anaerobic medium which consists of the following chemicals in distilled water. Sodium perchlorate, 1.00 g l^{-1} ; Sodium acetate, g l^{-1} or other C-sources as mentioned, 1 g l^{-1} and 10 ml $l^{-1}$of trace mineral supplement: 2.00 g l^{-1}; EDTA, 0.5 g l^{-1}; $MgSO_4$. $7H_2O$, 3.0 g l^{-1}; $MnSO_4$. H_2O, 0.5 g l^{-1}; NaCl, 1.0 g l^{-1}; $FeSO_4$. $7H_2O$, 0.1 g l^{-1}; $Co(NO_3)_2$. $6H_2O$, 0.1 g l^{-1}; $CaCl_2$ (anhydrous), 0.1 g l^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.1 g l^{-1}; $CuSO_4 \cdot 5H_2O$, 0.01 g l^{-1}; $AlK(SO_4)_2$ (anhydrous), 0.01 g l^{-1}; H_3BO_3, 0.01 g l^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.01 g l^{-1}; Na_2SeO_3 (anhydrous), 0.001 g l^{-1}; Na_2WO_4. $2H_2O$, 0.01 g l^{-1}; $NiCl_2 \cdot 6H_2O$, 0.02 g l^{-1}. The initial media pH was adjusted by adding the required amount of 1M H_2SO_4 or 1M NaOH solution. The enrichment medium was then transferred to 2.0 l conical flask crimp-sealed with butyl-rubber stoppers and needles.

Analytical Methods: Aliquot samples in the amount of 2 ml were taken using a needle and sterile syringes, centrifuged in 8000 rpm for 10 min, and kept in a refrigerator at 4oC if not analyzed same day. Perchlorate, nitrate, chlorate, sulfate, phosphate, and succinate were measured using a Metrohm 792 Basic Ion Chromatograph (Metrohm AG, Herisau, Switzerland) equipped with a Dual 3 column (250 mm × 4 mm), a RP guard column, and a conductivity detector. The detection limit for perchlorate was 0.5 ppm. Sodium hydroxide (5 mM) served as the eluent, and sulfuric acid (2.0 mM) as the regenerant. Sample volume was 20 µl. 100 mg $l^{-1}$of perchlorate solution was used to prepare perchlorate calibration

standards. Samples were filtered through the C-18 reverse-phase cartridge and then through 0.45µm filter. Ultra pure water was used for all analyses. Solution pH was measured using a Sartorius pH meter. Effluent phenol concentrations were ascertained using a colorimetric spectrophotometer (Odyssey DR/2500, Hach) and the 4-aminoantipyrine technique. Briefly, this method involved the reaction of phenols with 4-aminoantipyrine in the presence of potassium ferricyanide, to form a colored antipyrine dye. The dye was extracted from the aqueous dilutions of effluent samples with chloroform, and the color was measured at 460 nm. Samples were centrifuged at 8,000 rpm for 15 min to separate the yeast biomass. The supernatants were suitably diluted and COD analyses were carried out per standard methods [32].

Batch Degradation Study of perchlorate by *Pseudoxanthomonas* sp. using Phenol as Sole Carbon Source: All the biodegradation experiments with perchlorate and phenol were carried out in batch shake flasks of 150 mL Erlenmeyer flasks, containing 100 mL media with 5 mL of cell suspension (OD_{600} 0.15–0.25). The culture medium was incubated at 28^{o}C under shaking condition (120 rpm). The initial concentration of perchlorate and nitrate in the culture medium was varied from 100 mg/L to 800 mg/L with an interval of 100 mg/L. The optimum conditions for perchlorate degradation (pH and temperature) have been followed in the present study which has already been reported in our recent publication [33]. Samples were withdrawn at regular intervals, centrifuged (8000 rpm for 15 min) and filtered.

The supernatant was used to determine residual perchlorate and phenol concentration. The chemical oxygen demand (COD) of the supernatant, a measure of organic strength of wastewater, was determined by the dichromate method (closed reflux, titrimetric method) (APHA, 1998). After proper dilution, about 2.5 mL of the supernatant was added to the digestion solution (0.01667 M potassium dichromate) followed by addition of the sulfuric acid reagent. The whole mixture was digested at 150 ^{o}C for 2 h. Ferroin indicator (0.05 mL) was added to the digested mixture cooled at room temperature for titrating with standardized 0.01 M ferrous ammonium sulfate (FAS). The endpoint was determined by a sharp color change from blue-green to reddish-brown. The COD value was calculated as mg of O_2/L using the following equation

$$COD = (A-B) \times M \times 8000 / mL\ sample$$

Where A is the FAS used for blank (mL), B is the FAS used for sample (mL), M is the molarity of FAS and the factor 8000 is the milli-equivalent weight of O_2 x 1000 mL/L. The results reported in this work are the arithmetic average of the results obtained from the two repeated degradation experiments under identical conditions.

Degradation of Phenol as Sole C-source in Perchlorate Reduction by *Pseudoxanthomonas* sp. in Synthetic and Industrial Waste Water: The microbial culture of *Pseudoxanthomonas* sp. was shown to degrade phenol while removing perchlorate from water systems. This unique property of the mixed microbial consortium was used to simultaneously degrade the pollutants perchlorate and phenol.

An increasing concentration of phenol was added in batch shake flasks with a fixed amount of perchlorate (500 mg/L) to examine the capacity of the consortium to withstand phenol concentration. The anaerobicity of the culture was maintained by purging O_2-free N_2 in regular intervals. Phenol was added to the batch shake flask in the following concentrations, 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L, and 600 mg/L with the fixed amount of perchlorate in synthetic media. The consortium failed to grow beyond 600mg/L of phenol concentration in the previous experiment.

The removal of perchlorate, phenol and COD, and the growth were measured for each of the initial phenol concentrations in two replicates. The microbial consortium was again tested for removal of phenol present in real wastewater collected from oil refinery. The raw wastewater was quantified for some of its chemical and biochemical properties the wastewater was diluted accordingly to have the same range of initial phenol concentrations as in the previous case with synthetic wastewater. The removal of phenol, COD and growth of the culture were measured.

Reactor Set-up for Simultaneous Degradation of Perchlorate and Phenol: Two laboratory-scale up-flow packed bed bioreactor was designed for the effective perchlorate biodegradation by the enriched mixed culture consortium. Reactor start-up has been done for perchlorate degradation by the mixed consortium using phenol and succinate as C-source. The reactors' specifications are as follows: Column material: Plexiglas [Poly (methyl 2-methylpropenoate)], height: 34 cm, inner-diameter: 7 cm, volume: 1000 ml, Packing media: Polyurethane foam. Two upflow packed-bed reactor was simultaneously operated for perchlorate removal in constant temperature and pH. Reactor had same size, volume and packing media.

One was run for perchlorate degradation with phenol as the sole carbon source (R1) and another with phenol. To establish the reactor performance, the reactors were run for 30-45 days with a constant loading rate for start-up period after inoculating with an enriched mixed microbial consortium previously tested for perchlorate reduction. The degradation was monitored in regular intervals and growth of biofilm was observed during the start-up period. The biofilm was thoroughly generated in both the reactors after 15-20 days of start-up; both the reactors were operated for another 20-30 days in the same operating and environmental conditions to achieve a steady state.

RESULT AND DISCUSSION:

Perchlorate Degradation at Different Initial Concentrations: Batch experiments were conducted in different initial perchlorate concentrations (**Fig. 1**. Perchlorate initial concentrations were varied from 100 mg/L to 800 mg/L with interval of 100 mg/L. Phenol concentration was fixed at 500mg/L for all the initial perchlorate concentrations. Perchlorate was removed in all the initial perchlorate concentrations by the strain *Pseudoxanthomonas* sp. When the initial concentration was 800mg/L, the final concentration came down to 380 mg/L. After 9 days, the removal efficiency was 52.5%.

When the initial concentration was 700 mg/L, the final concentration came down to 100 mg/L. So the removal efficiency was 85.7%. For the initial concentrations 600 mg/L, 500 mg/L and 400 mg/L the final concentration came down to 100 mg/L after 9 days. The respective removal efficiency was 83%, 80%, and 75%. For 300 mg/L, 200 mg/L, and 100 mg/L the final concentration came down to 0 mg/L, so the removal efficiency was 100% in 9 days of batch culture. Therefore, it was observed that, with the decreasing initial concentrations, the overall removal efficiency have increased gradually from 52.5% to 100% with initial concentrations 800 mg/L to 100 mg/L, respectively.

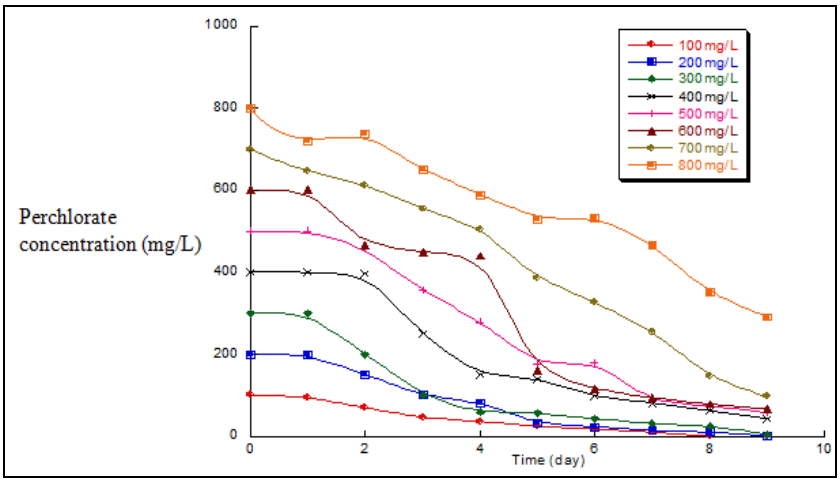

FIG. 1: DEGRADATION OF PERCHLORATE WITH VARYING INITIAL CONCENTRATION (MG/L) USING PHENOL AS A SOLE CARBON SOURCE

Growth Kinetics of *Pseudoxanthomonas* sp. Degrading perchlorate using Phenol as Sole Carbon Source: Batch cultures of the mixed consortium, growing on phenol as the sole-electron donor and perchlorate as the electron acceptor, were used in connection with the determination of growth kinetics **Fig. 2**. Only data representing the linear portion of the exponential growth were used

to calculate growth rates. The maximum observed growth rate of the culture was 0.145. Growth data were fitted by nonlinear regression analysis to obtain half-saturation constants of 1080 with phenol as an electron donor.

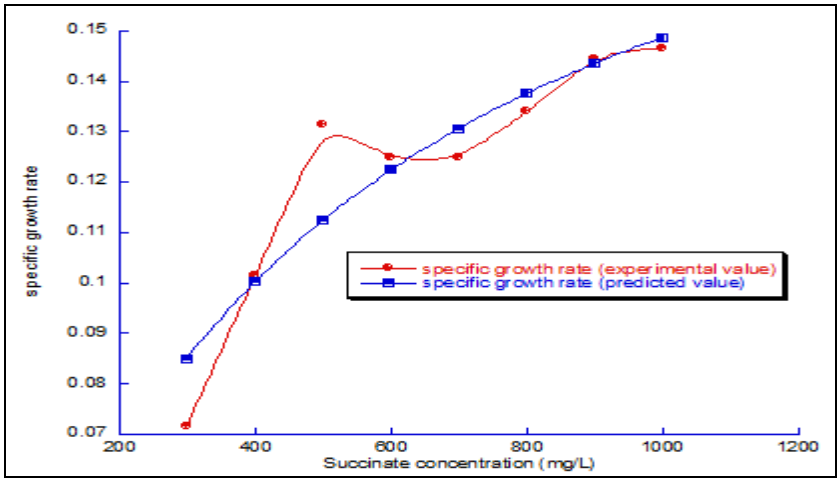

FIG. 2: GROWTH RATES OF *PSEUDOXANTHOMONAS* SP. DEGRADING PERCHLORATE USING PHENOL AS THE SOLE ELECTRON DONOR

Degradation of Perchlorate using Phenol as Sole Carbon Source in Synthetic and Industrial Waste Water in Batch System: Phenol was added in different concentrations with fixed perchlorate concentration (500 mg/L) in the culture media. The microbial consortium has shown to degrade phenol upto 600 mg/L initial concentration.

Beyond 600 mg/L, the microflora almost ceases to grow and stops the degradation of phenol as well as perchlorate. The degradation rate was decreased with increasing initial phenol concentration **Fig. 3A** and **3B**.

It has followed the conventional inhibition kinetics of phenol. The overall degradation of phenol was faster in the real wastewater collected from a petroleum refinery, indicating the presence of indigenous phenol degrading microflora. The lag period of microbial growth in the synthetic wastewater was more than the time required in the case of industrial wastewater **Fig. 4A** and **4B**.

The wastewater was collected microbe from oil refinery and likely to have phenol degrading indigenous, like *Pseudomonas* sp., which would possibly enhance the degradation function of the microcosm. Perchlorate was degraded in a substantial amount. In a lower initial concentration of phenol (50 mg/L) only 60% of perchlorate was removed. It is very clear from the figures that the rate of perchlorate degradation has increased with the increased phenol concentration.

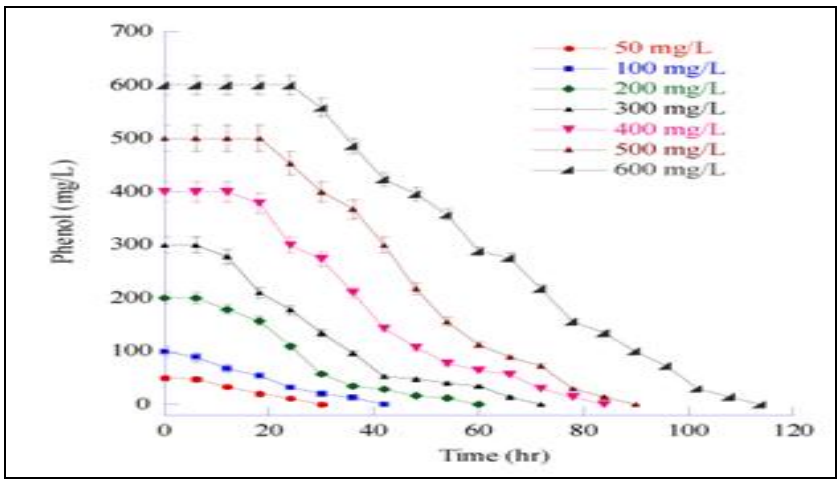

FIG. 3A: PHENOL DEGRADATION IN SYNTHETICWASTE WATER

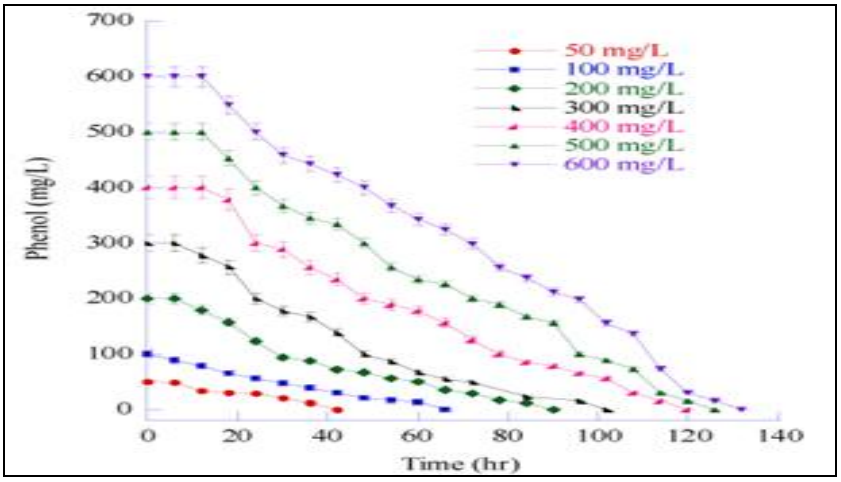

FIG. 3B: PHENOL DEGRADATION IN SYNTHETIC WASTEWATER

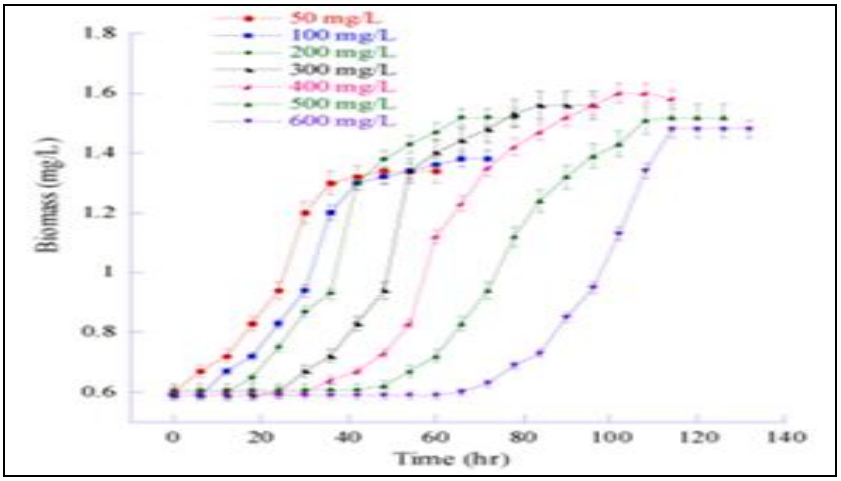

FIG. 4A: MICROBIAL GROWTH IN SYNTHETIC WASTEWATER

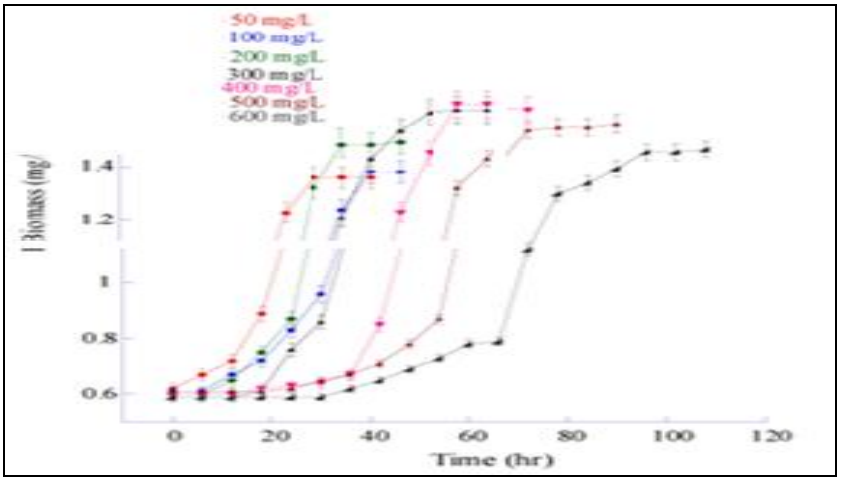

FIG. 4B: MICROBIAL GROWTH IN INDUSTRIAL WASTEWATER

Model-fitting for Degradation Phenol by *Pseudoxanthomonas* sp. in Batch System: In our present study, we have fitted the experimental data obtained from the batch degradation experiments (described in Section 2.4) to several kinetic models (such as, the Haldane model, Yano model, Aiba model and Webb model) to represent the degradation kinetics of phenol in order to select the suitable model(s). The degradation rate, q (1/h) for those models are represented in **Table 1**. Where, S_o is the initial substrate concentration (mg/L), q_{max} is the maximum degradation rate (1/h), K_s is the substrate-affinity constant (mg/L), K is the constant in Yano and Webb models (mg/L) and K_i is the

substrate-inhibition constant (mg/L). A larger K_i value indicates the culture is less sensitive to the substrate inhibition. The degradation rate, q was determined from the gradient of a semi-logarithmic plot of substrate concentration S vs. time for each initial substrate concentration. From the values of q vs. S_o, the values of the kinetic parameters for various models were obtained using nonlinear regression analysis in SOLVER on MS-Excel (Microsoft Corporation). The relation between the degradation rate and the initial substrate concentration is represented by a set of empirically derived rate equations referred to as theoretical models generated to describe the behaviors of a given system. The figure also shows the predictions of the experimental data by the three different models **Table 1** used in this work. The experimentally obtained substrate degradation rate values at various initial concentrations were used to fit the above models for estimating the kinetic parameters using the nonlinear regression analysis in SOLVER on MS-Excel, Microsoft Corporation. The values of the biokinetic parameters based on the fitting of the above models are reported in **Table 1.** The Yano model predicts the experimental degradation data fairly well for the pollutants, perchlorate and nitrate. It gives the best fit with a coefficient of determination, R^2 = 0.906 respectively with SD_{avg} = 0.007782976 **Table 1**. Estimation of these biokinetic parameters is highly sensitive to their initial guess values required as input during regression analysis. Improper initial guess values may result in inaccurate values of the parameters, which in turn change the accuracy of the prediction of experimental degradation profiles. Therefore, we have carried out a sensitivity analysis to estimate these parameters in this study. Here, three different sets of biokinetic parameters (q, K_s, and K_i) values were used as initial guesses, as indicated in **Table 2**. Degradation profiles were simulated by applying all three models using the estimated biokinetic parameters for the different sets of initial guess values. The coefficient of determination (R^2) for each case was determined. The R^2 values, thus obtained by various initial guesses considered, are reported in **Table 1**. It can be observed from the R^2 values in the table that the Webb model has the most consistency for both the pollutants. The Yano model provides a better fit for perchlorate, as can be seen from **Fig. 6** as well as from the R^2 values in **Table 2**. The Webb and Yano models show similar R^2 values for the various initial guess values with negligible deviations for nitrate except for Case 2 of the Yano model, where initial guess values were $q < 1$, $K_s > 1$, $K_i > 1$ **Table 2.**

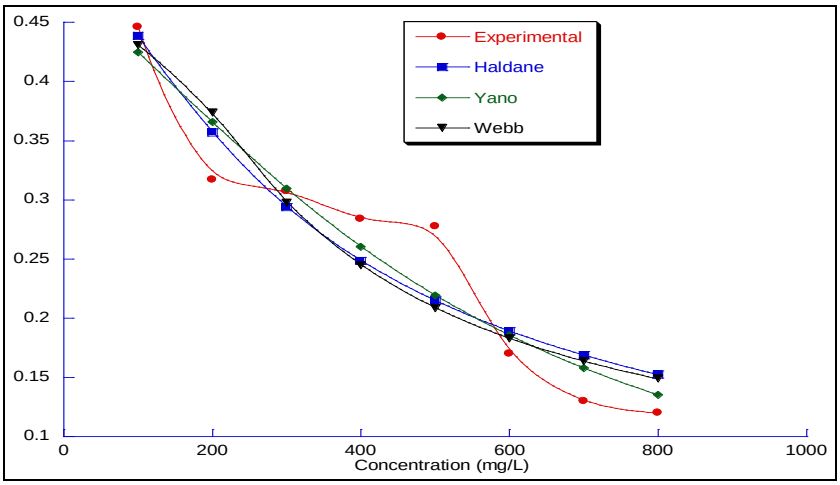

FIG. 5: EXPERIMENTAL AND PREDICTED DEGRADATION RATES OF PHENOL DEGRADED BY *PSEUDOXANTHOMONAS* SP.

TABLE 1: CALCULATED KINETIC CONSTANTS FOR THE THREE MODELS MOST FITTED TO THE PHENOL DEGRADATION

Initial value	R^2 value		
	Haldane Model	Yano Model	Webb Model
Case 1: $q < 1$, $K_s < 1$, $K_i < 1$	0.884	0.9057	0.8685
Case 2: $q < 1$, $K_s > 1$, $K_i > 1$	0.88	0.9056	0.8685
Case 3: $q = 1$, $K_s > 1$, $K_i > 1$	0.883	0.9051	0.8685
Case 4: $q > 1$, Ks < 1, KI < 1	0.8837	0.9052	0.8685

TABLE 2: INITIAL GUESS VALUES AND THE CORRESPONDING COEFFICIENT OF DETERMINATION (R^2)

Pollutant	Model	q_{max} (1/h)	K_s (mg/L)	K_I (mg/L)	K (mg/L)	R^2	SD_{avg}
Phenol	Haldane (Haldane, 1965) $q_{max}S_o\ K_s+S_o+(S_o^2/\ K_i$	0.72	17.1	218.08		0.8837	0.0134
	Yano (Yano *et al.*, 1966) $q_{max}S_o\ \ K_s+S_o+(S_o^2/K_i)$ $[1+(S_o/K)]$	0.49	1	915.19	406.55	0.9060	0.0138
	Webb (Webb, 1963) $q_{max}S_o[1+(S_o/K)]\ S_{o+}\ K_s+(\ S_o^2/K_i)$	7.8	136.8	11.849	2408.3	0.8602	0.0193

Influence of Different Co-pollutants Affecting Perchlorate Degradation using Synthetic Waste Water in Batch System: The anions like nitrate, nitrite, chlorate, and phosphate are used in several industries, especially fireworks, fertilizer, electroplating electro-polishing and army ammunition along with perchlorate and therefore were known to co-exist in waste streams originating from these industries. Among them, nitrate is a common co-contaminant, and microbiological perchlorate reduction in many systems has been found to be reduced or inhibited in the presence of nitrate. The effect of different co-anions on perchlorate reduction by the enriched mixed consortium is depicted in **Fig. 1,** which shows the perchlorate degradation was affected in the presence of these co-anions. In the medium containing nitrate and perchlorate together at the same concentration (500 mg L^{-1}), degradation started without any lag phase in a mixture containing perchlorate and nitrate **Fig. 6**.

The decrease in perchlorate concentration during the first 48 hrs was supported by an increase in the cell density ($O.D_{600}$) from 1.0 (t = 1 h) to 1.2 (t = 48 h). However, its degradation efficiency was only 47% at the end of six days and did not improve further. Several studies have reported the influence of nitrate on perchlorate degradation by denitrifying perchlorate reducers [34]. The inhibition of perchlorate degradation in the presence of nitrate is attributed mainly to the suppression of (per) chlorate reductase by nitrate [35]. However, the existence of separate pathways for the two e^- acceptors has also been proposed [36]. The preference of ClO_4^- to NO_3^- as electron acceptor is likely to be associated with a different enzyme involved, which lowered the activation energy [37]. The mixed consortium almost completely degraded nitrite within 1 day of incubation time **Fig. 6**. The degradation rate of nitrite was higher than the nitrate in the presence of perchlorate as observed in the previous experiment. No nitrite was found to accumulate in the media in the presence of nitrate, and nitrite was completely reduced, so it can be stated from the result that the mixed consortium was able to complete the denitrification process starting from nitrate to nitrite and then to gaseous nitrogen. The higher rate of nitrite reduction than nitrate reduction is also supported by the lower Gibb's free energy value required for nitrite reduction than nitrate reduction. It can also be inferred that the perchlorate reducing mixed consortium can withstand a sufficiently high amount of nitrogen, whereas in a study by Bardiya and Bae [38] with indigenous mixed culture 100 mg L^{-1} was found to be toxic for the culture. The reduction rate was also affected in the presence of chlorate (ClO_3^-) **Fig. 6**. Chlorate was reduced in a much faster rate than perchloarate. The consortium was able to degrade perchlorate up to ~57 %, while chlorate was degraded up to ~81 % (after 6 days of incubation).

It has been reported that (Per)chlorate reducing bacteria (PCRB) use a single enzyme (per)chlorate reductase for the degradation of perchlorate (ClO_4^-) to chlorate (ClO_3^-) and chlorate to chlorite (ClO_2^-) [12]. Chlorite (ClO_2^-) is then converted into chloride (Cl^-) and molecular oxygen by the enzyme chlorite dismutase [39,40]. Therefore observed effective degradation of perchlorate with uptake of chlorate by the mixed microbial culture in the study is not unlikely. In the presence of phosphate, although the culture utilized both the anions simultaneously in a substantial rate by the enriched mixed culture **Fig. 6**. For growth 1.5 OD_{600} in 6 days, the enriched mixed consortium sufficiently utilized these two e^- acceptors. However, the perchlorate degradation efficiency was low at ~54% (after 6 days) compared to media containing only perchlorate. The decreased degradation of perchlorate in the presence of other co-pollutants could be analyzed by using phenol in each system.

The results found that phenol was utilized almost fully by the mixed consortium after 6 days in each case, which affected the degradation of perchlorate. This insufficient C-source hindered the further perchlorate degradation by the enriched mixed culture.

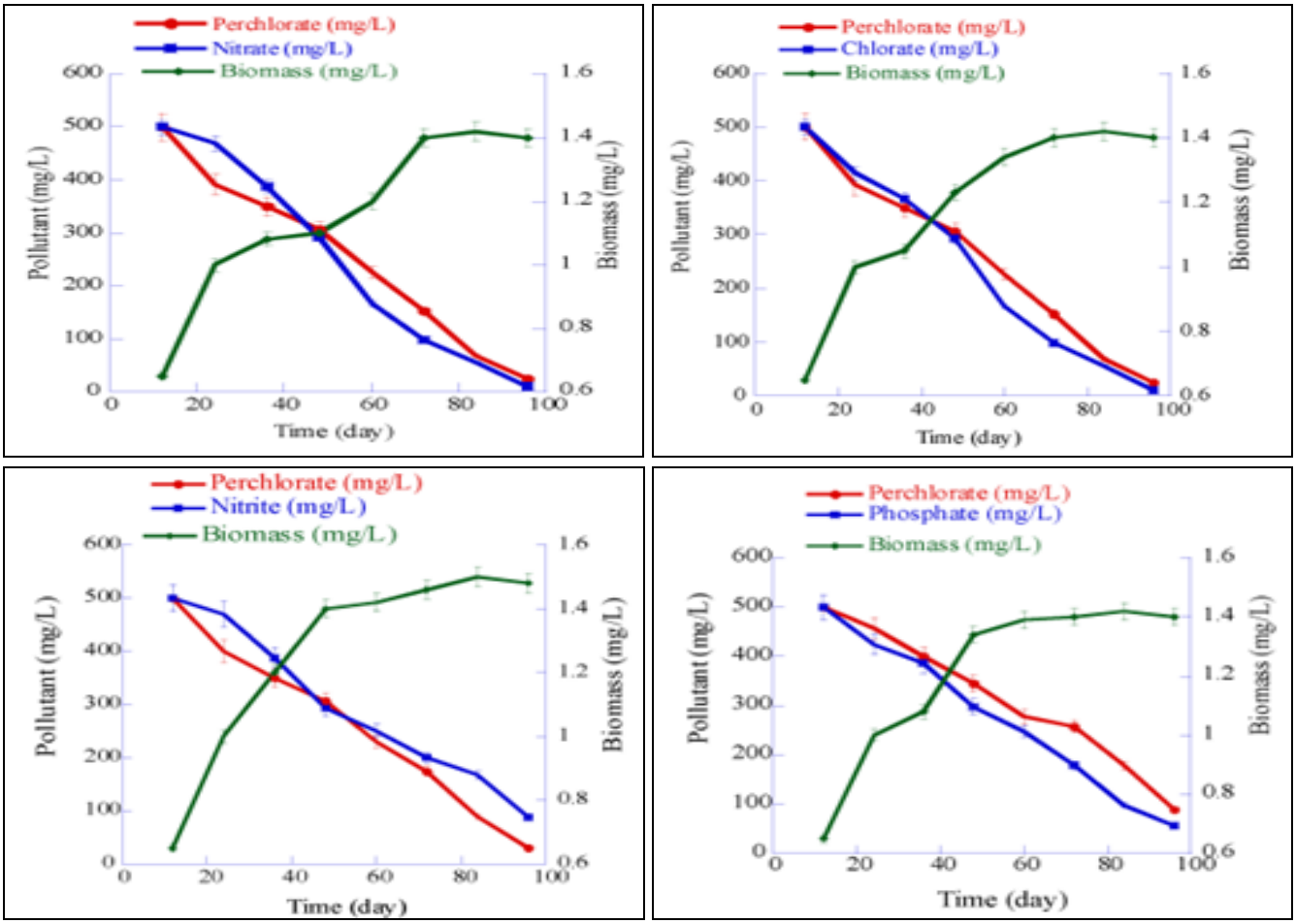

FIG. 6: DEGRADATION OF PERCHLORATE IN THE PRESENCE OF OTHER CO-POLLUTANTS USING PHENOL AS SOLE C-SOURCE ALONG WITH MICROBIAL GROWTH PATTERN

Performance of the Continuously Operated Packed Bed Bioreactor Degrading Perchlorate using Phenol and Succinate as a Sole Carbon Source by *Pseudoxanthomonas* sp: In stage-I, Degradation performances of R1 were examined in different stages, varying the hydraulic retention time (HRT). Both the reactors were subjected to start with 10 days HRT with 200 mg/L of perchlorate and 400 mg/L of succinate as C-source. Gradually the HRT was decreased for both the reactors from 10 days to 3 days. The flow rate was changed when the removal efficiency reached a steady-state **Fig. 7** and **Fig. 8**. In stage II, reactor R1 and R2 were run in 400 mg/L of perchlorate and 800 mg/L of C-source, and again HRT was reduced from 10 days to 3 days, changing the flow rate after reaching a steady-state **Fig. 7** and **Fig. 8**. In stage, I, both the reactors had shown to reach a steady-state within 5 to 6 days from the starting period when the HRT was set at 10 days. When the HRT was changed from 10 to 7 days, the removal efficiency for perchlorate and COD came down to 20 %. To decrease the HRT further from 7 days, the flow rate was adjusted to make HRT 6 days for 4 to 5 days for both the reactors, and then HRT was adjusted to 5 days. Likewise, when further decreasing the HRT from 5 days to 3 days, both the reactors were run in HRT 4 days for 3 to 4 days. Both the reactors have shown up to 96% to 100% removal efficiency for perchlorate when a steady-state has been reached. In HRT 10, 7, 5, and 3 days, flow rates were changed when steady states were observed for 6 to 8 days for perchlorate and COD removal. In stage II, perchlorate and COD concentrations were doubled, and both the reactors operated at increasing HRT from 10 days to 3 days as in stage I. In both the reactors, more time was required to attain the steady-state when HRT was

10 days. HRT was gradually decreased from 10 to 9, then 8 and then 7 days. Unlike stage I, in stage II, both the reactors took 3 to 4 days to attain the steady state every time when the HRT was changed. In both the reactors steady state was achieved with 100% and 96% removal efficiency for perchlorate and COD. In stage III, HRT was again reduced, phenol was gradually added increasingly with succinate keeping the HRT at 2 days **Fig. 8, 9, 10, 11**. The reactor was run with the same initial influent concentration, 400 mg/L of perchlorate and 800 mg/L phenol. When HRT was reduced to 2 days the steady-state came after 11 days of running the reactor. After that, unlike stage II, the effluent concentration got increased and again decreased to reach a steady state within 10 days. Again, when HRT was made for 1 day same phenomenon was observed with less increase in effluent perchlorate and phenol concentration during the phase of acclimatization. When HRT was changed to 12 h from 1 day, the effluent pollutant concentration was increased and observed not to be decreased for 5 to 6 days. The HRT was increased upto 18 h, and a steady state was achieved within 10 days of operation. The removal efficiency was observed to be decreased when HRT was changed from 1 day to 12 h and so on. The same acclimation phase was observed before reaching the steady-state when HRT was changed to 16 and 14 h. Still, the increase in effluent concentration for both perchlorate and phenol was observed to be gradually reduced the efficiency remained the same for perchlorate (97%) and phenol (96%). In this stage, the reactor was run with the same HRT (2 days) and initial perchlorate 400 mg/L, succinate 750 mg/L and phenol 50 mg/L **Fig. 9**. Gradually succinate was replaced by phenol in the following sets of operations. It was observed that the effluent concentration of perchlorate, phenol and succinate was increased and then decreased within 10 days after changing the loading rate; the acclimation phase showed similar nature as R1 while increasing the flow rate. The phenol concentration was gradually increased from 50 to 100 and then to 200 mg/L. While increasing the phenol concentration upto 400 mg/L, the removal efficiency showed to be 10 to 15 % for both perchlorate and phenol **Fig. 11** and **Fig. 12**. The effluent concentration was observed not to be decreased for 8 to 10 days. The phenol concentration was decreased upto 350 mg/L, and the removal efficiency increased within 3 to 4 days and reached a steady state for both perchlorate and phenol removal.

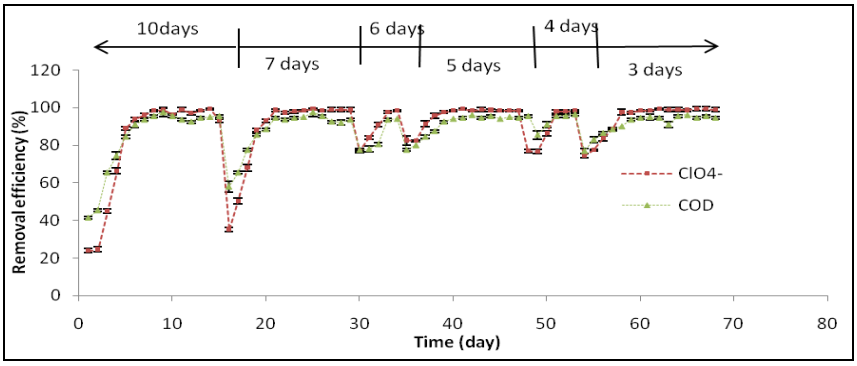

FIG. 7: PERFORMANCE OF R2 SHOWING % REMOVAL OF PERCHLORATE (200MG/L) AND SUCCINATE (400 mg/l) IN DIFFERENT HRT

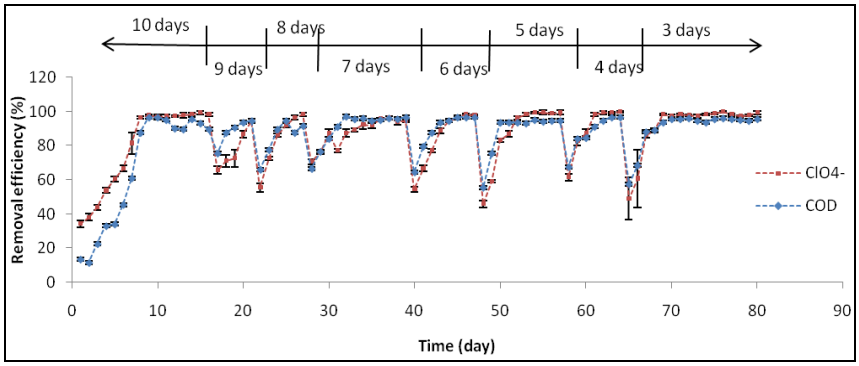

FIG. 8: PERFORMANCE OF THE R2 SHOWING % REMOVAL OF PERCHLORATE (400MG/L) AND SUCCINATE (800 mg/l) IN DIFFERENT HRT

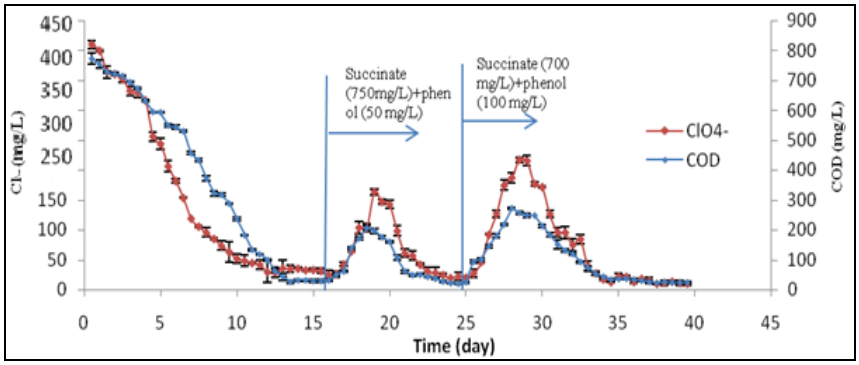

FIG. 9: PERFORMANCE OF THE R2 SHOWING EFFLUENT PERCHLORATE (400mg/l) AND IN DIFFERENT SUCCINATE AND PHENOL CONCENTRATION IN HRT 2 DAYS

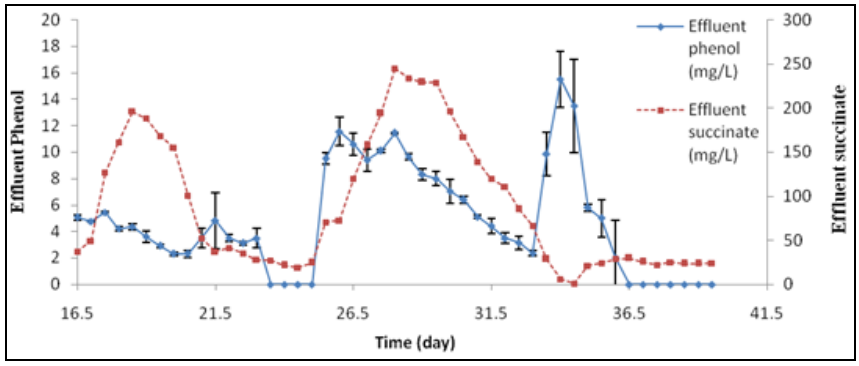

FIG. 10: PERFORMANCE OF THE R2 SHOWING EFFLUENT PHENOL AND SUCCINATE IN HRT 2 DAYS

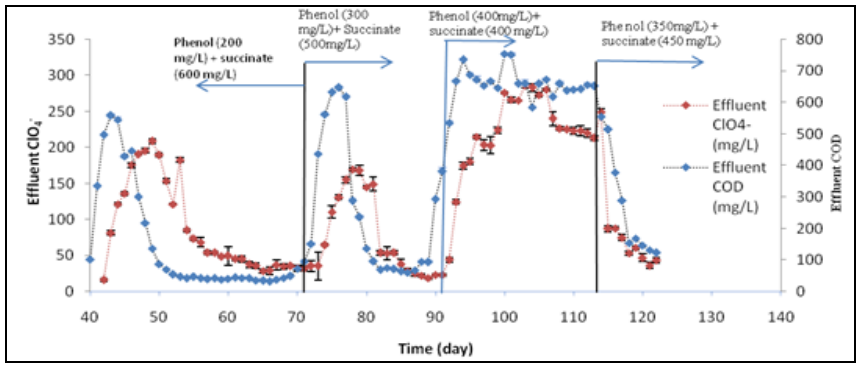

FIG. 12: PERFORMANCE OF THE R2 SHOWING EFFLUENT PHENOL AND SUCCINATE IN HRT 2 DAYS

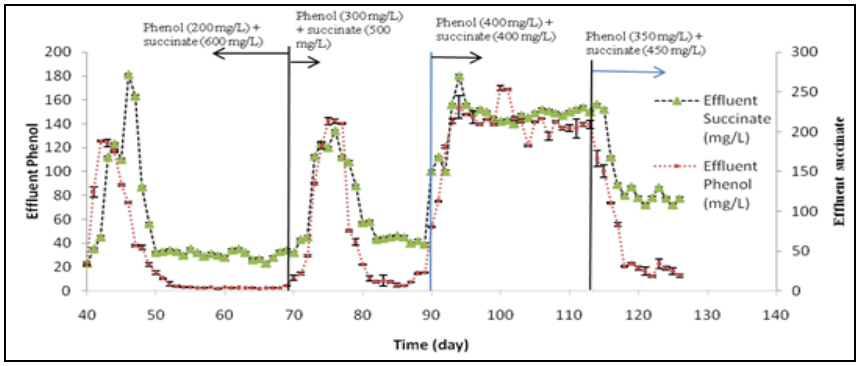

FIG. 11: PERFORMANCE OF THE R2 SHOWING EFFLUENT PERCHLORATE (400mg/l) AND COD (PHENOL AND SUCCINATE) IN HRT 2 DAYS

CONCLUSION: The results obtained from this experiment show the bright possibility of using the microcosm in the biological treatment of wastewaters with a considerably high concentration of phenolics and simultaneous reduction of perchlorate from the water system. The microorganism, *Pseudo-xanthomonas sp.* was found to degrade phenol and perchlorate simultaneously for the first time. This novel finding explores the prospects of further research that can

be employed broadly in wastewater treatment technology.

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CONFLICTS OF INTEREST: None

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