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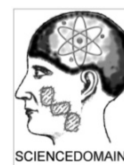

Simultaneous Carbon-Oxidation and Nutrient (N and P) Reduction in a Batch-Fed Reactor by *Pseudomonas sp.* P1 Isolated from a Small-scale Slaughterhouse Wastewater

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Authors' contributions

This work was carried out in collaboration between all authors. Author SM designed and supervised the investigation. Authors VD and PK carried out the experimental study. Author VD did the literature searches. Author PK performed the kinetic analysis and wrote the first draft of the manuscript. All authors perused and approved the final manuscript.

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ABSTRACT

Aims: Laboratory scale evaluation of phosphorus removal along with organic and nitrogen reduction in a batch reactor under anoxic-aerobic environment using Phosphorous Accumulating Organisms (PAOs).
Study Design: Isolation of the predominant bacterial species through phenotypic and phylogenetic characterization of acclimatized sludge collected from a slaughterhouse wastewater treatment plant. Selected isolates able to utilize acetate compounds as superior carbon and energy source in sequential mode of operation.
Place and Duration of Study: Environmental engineering Laboratory, Department of Civil

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Engineering, Jadavpur University, Kolkata, India from March 2013 until October 2015.
Methodology: Biological phosphorous study was performed in presence of acclimatized PAOs under anoxic and aerobic condition environment in a batch fed reactor. Selection and Isolation of the most potent isolate and identify PAO species by 16S rDNA study. Thereafter, kinetics study of phosphorous reduction in presence of organic carbon and nitrogen source along with some important process parameters affecting the performance.
Results: Molecular phylogenetic identification, supported by chemotaxonomic and physiological properties assigned isolated PAOs (*Pseudomonas sp.* P1) as a close relative (99%) of *Pseudomonas aeruginosa*. Isolated PAOs (*Pseudomonas sp.* P1) showed that 94.30% SCOD reduction from an initial SCOD value of 600±20 mg/L and 81.38% and 87.98% of NH_4^+-N and PO_4^{3-}-P removal from an initial NH_4^+-N and PO_4^{3-}-P concentration of 50±5 and 10±1 mg/L, after 24 hr contact period in a batch reactor.
Conclusion: The investigation result demonstrated that the bacterial species *Pseudomonas sp.*P1 exhibited appreciable removal of phosphorous along with organic (SCOD), nitrogen (NH_4^+-N, NO_3^--N) in the batch reactor under anoxic and aerobic condition sequentially which may be applied for real life situation particularly to treat slaughterhouse effluent.

Keywords: Biological phosphorus removal; organic reduction; nitrogen reduction; pseudomonas sp. P1; slaughterhouse wastewater.

1. INTRODUCTION

Wastewater containing nitrogen and phosphorus along with organisms when enter the environment in exceeding quantity, the freshwater ecosystems suffer disastrously. Nutrient pollution in streams, rivers, lakes, bays and coastal water leads to serious environmental threats and human health which affects the economy of the country.

Elevated amounts of nitrogen and phosphorus in the water cause eutrophication and enhanced rate of algal growth. Profuse growth of algae harms water quality, food resources and habitats, and decreases the oxygen that fish and other aquatic life need to survive. Large growths of algae are called algal blooms and they can severely reduce or eliminate oxygen in the water, leading to illnesses in fish and the death of large numbers of fish. Some algal blooms are harmful to human because they produce elevated toxins and bacterial growth that can make people sick if they come into contact with polluted water, consume tainted fish or shellfish, or drink contaminated water. In addition to that it causes odour problem in the water bodies. Nitrate in drinking water causes methaemoglobinemia (Blue Baby syndrome) in infants while excess phosphates may lead to gastric problems. In freshwater systems, phosphorus is typically serves as a nutrient that relatively less supply to biological needs, which means that the productivity of aquatic plants and algae can be controlled by limiting the amount of phosphorus entering the water. The primary sources of these nutrients in water bodies are agricultural run-off, storm water, municipal wastewater and effluent from industries like pharmaceuticals, detergent, toothpaste, brewery and small scale units like slaughterhouse and meat-processing units.

Biological phosphorus removal (BPR) is traditionally accomplished in alternate anoxic and aerobic conditions which favours for the growth of Phosphorous Accumulating Organisms (PAOs) that are primarily responsible for the phosphorous removal from the wastewater if the supply of Volatile Fatty Acids (VFAs) in the feed stock is maintained [1]. Earlier studies have shown that BPR could be achieved by microbial community like PAOs among which the *Proteobacteria* beta subclass was found to be the most dominant species [2]. Later on both *Acinetobacter sp.* and *Pseudomonas sp.* were also found to be the major groups of PAOs responsible for BPR [3]. Some PAOs such as *Acinetobactor sp., Pseudomonas sp., Moraxella sp., Aeromonas sp.,* etc. have also exhibited earlier to accumulate poly-P aerobically in activated sludge [4]. An initial anoxic/anaerobic zone allows the PAOs to take up VFAs into their cells and store them in the form of polyhydroxyalkanoates (PHA) for which there must be a supply of short-chain fatty acids as the carbon source and a source of energy to bind them together into PHA which comes from the hydrolysis of poly-P like ATP in the cell and the resulting orthophosphates are released into the environment. In the subsequent aerobic condition, when there is a lack or absence of readily biodegradable COD (rbCOD), the stored

PHB is used by the PAOs for cell growth and this PHB oxidation releases a high amount of energy (24-36 times more than in anaerobic phase) which is utilized for reforming polyphosphates in the cell from all the available orthophosphate in the surrounding and also for the synthesis of polyglucose (glycogen). The PAOs thus collect a greater amount of phosphorous in the aerobic part of the cycle after which a number of cycles, a majority of the orthophosphate is incorporated into the biomass which ultimately separated from the wastewater by settling and filtering the sludge.

Some earlier studies demonstrated that the removal rate of phosphate was influenced by a number of factors such as organic loading , anoxic and aerobic period, different varieties of carbon source in the feed, mixed liquor volatile suspended solid (MVLSS) concentration, pH, temperature, dissolved oxygen (DO) concentration and the type of sludge used in the system i.e. flocculate or granulate [5-10]. Kargi and Uygur [11] studied the Nutrient removal from synthetic wastewater by sequencing batch operation at different specific nutrient loading rates (SNLR) obtaining the highest COD (99%), NH_4^+-N (99%) and PO_4^{3-}-P (97%) removal efficiencies with the initial COD concentration of 600 mg/l ,at COD loading rate of nearly 40 mg COD/(g biomass)/hr. A separate lab-scale SBR study was conducted by Obaja et al. [12] to explore the feasibility of using an internal carbon source (non-digested pig manure) for biological nitrogen and phosphorus removal in treating digested piggery wastewater. The internal carbon source used for denitrification could have similar effects to acetate. The study showed 99.8% of nitrogen and 97.8% of phosphate were removed in the SBR, from an initial concentration in the feed solution of 900 mg/L ammonia and 90 mg/L phosphate. Pala and Bölükbas [13] subsequently investigated the biological carbon, nitrogen, and phosphorous removal in a municipal wastewater treatment plant (MWWTP) using batch tests. Average phosphorus release rate was 0.019 mg P/g VSS min for glucose addition and 1.63 mg P/g VSS min for acetate addition. The average P uptake rates were 0.0031 and 0.0046 mg P/g VSS min, for aerobic and anoxic conditions respectively. In a separate study, an anaerobic/aerobic/anoxic process (referred to as an AOA process) using a Sequential batch reactor (SBR) was proposed by Tsuneda et al. [14] for simultaneous phosphorus and nitrogen removal from wastewater. The AOA process was operated under stable condition for more than a year when a certain amount of carbon substrate (40 mg-C/L in a reactor) was supplemented to inhibit aerobic phosphate uptake. The average nitrogen and phosphorus removal efficiencies were found to be 83% and 92%, respectively. It was demonstrated that phosphate-accumulating organisms (PAOs) capable of utilizing nitrite as an electron acceptor, the so-called denitrifying phosphate-accumulating organisms (DNPAOs), could exist in the AOA process. Simultaneous biological phosphorus and nitrogen removal with enhanced anoxic phosphate uptake was further investigated by Lee et al. [15] in an anaerobic–aerobic–anoxic–aerobic sequential batch reactor [$(AO)_2$-SBR]. The ratio of the anoxic to the aerobic phosphate uptake capacity was increased from 11 to 64% by creating an anoxic phase in the SBR. Average removal efficiencies of TOC, total nitrogen, and phosphorus were found to be 92, 88, and 100%, respectively. Nittami et al. [16] studied the influence of temperature, pH and DO concentration on EBPR performance under fully aerobic conditions in SBR which showed that P release and uptake occurred during every aerobic cycle where environmental conditions were varied from the standard parameters except when the pH was changed where the net P removal deteriorates over a short time period at pH around neutral.

The objective of the present study was to evaluate performance of a laboratory-scale batch reactor for combined removal of soluble organics and phosphorous along with nitrification and denitrification kinetics using synthetic feed solution keeping in view how different influencing parameters such as initial MVLSS concentration, initial phosphate level, initial soluble chemical oxygen demand (SCOD), presence of acetate and presence of nitrate affect the phosphorus removal. The study also includes isolating the predominant bacterial species from acclimatized sludge by morphological, physiological, biochemical examinations along with 16S rDNA study.

2. MATERIALS AND METHODS

2.1 Seed Acclimatization

The microbial seed was cultured by mixing digested sludge collected from a slaughterhouse wastewater treatment plant in an aspirator bottle used as digester of 3L capacity. The seed was allowed to grow in alternate 24 hr of anoxic and aerobic condition by transferring the seed from the anaerobic aspirator bottle to an aeration tank

where aeration was done by means of diffused air system using aquarium pumps. The pH in the reactor was maintained in the range of 7.0-7.5 depending upon the anoxic/aerobic condition. The acclimatization phase was continued until a steady state condition was observed that is equilibrium SCOD and Phosphate removal vis a vis steady growth of MLVSS concentration. The targeted feed solution composition included in the study SCOD ($C_6H_{12}O_6$ and CH_3COONa), 500 mg/L; NH_4^+-N (NH_4Cl), 50 mg/L; PO_4^{3-}-P (KH_2PO_4 and K_2HPO_4), 10 mg/L; NO_3^--N (KNO_3), 10 mg/L and trace element solution, 5.0 ml/L. The composition of the trace element solution was $FeCl_3{\cdot}6H_2O$, 710 mg/L; $CuSO_4.5H_2O$, 0.1 mg/L; $CaCl_2{\cdot}2H_2O$, 100 mg/L; $CoCl_2{\cdot}6H_2O$, 50 mg/L; $ZnSO_4{\cdot}7H_2O$, 0.1 mg/L; H_3BO_3, 150 mg/L; and $(NH4)_6Mo_7O_{24}$, 0.11 mg/L.

2.2 Isolation of the Bacterium and Development of Pure Culture

One mL of mixed acclimatized liquor containing poly-P-bacteria was taken from aspirator bottle and mixed with 9mL of distilled water in a test tube, thereby diluting the sample 10^{-1} times. Then a serial dilution of the sample was carried out up to 10^{-9} dilution by taking 1 ml sample from each diluted sample and diluting it 10 times with distilled water. Then, 100 µL solutions was taken from the 10^{-9} diluted sample and spread on nutrient agar (NA) petri plate and was finally incubated at 37℃ for 24 hr. The composition of 500 mL nutrient agar medium are beef extract, 1.5 gm; Peptone, 2.5 gm; sodium chloride, 2.5 gm; agar, 8 gm; pH = 7.0-7.2. The contents of nutrient agar medium were taken in a 1000 mL beaker and mixed well with 500 mL distilled water. Then the medium was sterilized in an auto-clave under saturated steam condition at 121℃ temperature for 20 mins. Then 10 ml of sterile nutrient agar medium was poured into sterile petridishes and kept for solidifying inside a laminar bench. After 24 hr of incubation, growth was observed on the NA plates and the most predominant colony present among others were isolated and streaked into a separate NA plate and incubated at 37℃ for 24 hr. When single type colony was observed that grown on the plate, it is said to be a dominant pure culture of bacteria isolated from the mixed sludge.

2.3 Molecular Phylogenetic Analysis of Isolate P1

Genomic DNA was extracted following the procedure as describe by Sambrook et al. [17]. The 16S rRNA gene of the bacteria was amplified with forward primer 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and a reverse primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') [18]. The PCR amplification has been done in a 50 µL reaction mixture containing 5 µL of 10X PCR buffer with 1.5 mM $MgCl_2$, dNTP 4 µL, 50 pmol of each oligonucleotide primers, 3U *Taq* DNA polymerase 1 µL. 10 µL of isolated genomic DNA was added to the mixture and incubated in a thermal cycler (Mastercycler personal; Eppendorf AG) for an initial denaturation of 3 min at 94℃ followed by 30 incubation cycles each consisting of 1 min denaturation at 94℃, 30 sec annealing at 52℃, 2 min elongation at 72℃ and a final 7 min elongation at 72℃. Amplified DNAs were purified by the spin column method (WIZARD PCR Prep DNA Purification System, Promega). Templates from the isolates were sequenced with an Applied Biosystems 3730xl Genetic Analyzer through an outsourcing agency SciGenom Labs Pvt. Ltd. Situated at Kakkanad ,Cochin, Kerala, India. The same PCR primers were used to sequence the templates**.** Sequence data were compiled with the BioEdit program (http://www.mbio.ncsu.edu/ BioEdit/ bioedit.html) and examined for sequence homology with the archived 16SrDNA sequences from GenBank at www.ncbi.nlm.nih.gov/nucleotide, employing the BLAST search program [19]. Multiple sequences were aligned with CLUSTAL W [20]. Phylogenetic analyses were performed according to the neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP* version 4b10 [21].

2.4 Determination of Physiological Characteristics of Isolate P1

Morphological and physiological characteristics of the isolate P1 were determined following the Bergey's manual [22]. Presence of flagella, anaerobic growth, sodium chloride tolerance, indole production, catalase activity, gelatin liquefaction, deoxyribonuclease activity, Voges-Proskauer test, methyl red test, starch hydrolysis, β-galactosidase activity, urease test and starch hydrolysis were carried out following the methods described in Kundu et al. [23]. Nitrate reduction test was performed to determine whether the isolate P1 elaborated the enzymes nitrate reductase and nitrite reductase. These two enzymes catalyze two reactions required for converting nitrate to the end product as nitrogen gas. Aliquot from a pure culture of the isolate P1 was aseptically inoculated to a sterile tube containing nitrate broth with an inverted

Durham's tube. The inoculated tube was incubated at 37℃ for 24 hrs. Finally the reductions of nitrate and nitrite were ascertained by addition of naphthylamine and sulphanilic acid solutions to the nitrate broth followed by adding a small amount of zinc dust [24]. Acid production from various carbohydrates was done following Hinz et al. [25], utilization of sole carbon, nitrogen and amino acids was done according to Gutnick et al. [26] while growth in various media, different temperatures and pH as well as phosphorous utilization test were done in accordance with standard procedures as follows. To determine the optimum temperature and pH for growth, the isolate was cultured in Luria-Bertani medium and incubated at 37℃ with shaking (100 rpm) for 48 hr. Bacterial growth was confirmed by measuring the optical density of the culture in UV-Visible spectrophotometer at 600 nm. Lysine and arginine decarboxylase activities were measured following Møller, [27] whilst the production of flexirubin pigment was tested according to Fautz and Reichenbach [28]. All the above tests were done in triplicate sets.

2.5 Methodologies for Evaluation of Removal Kinetics by Isolate P1

A basal inorganic medium (synthetic wastewater) was prepared for the enrichment and evaluation of removal of combined SCOD and nutrient (P and N) by the isolated Phosphorous Accumulating Organism (PAO) in P1 strain. The composition of basal medium was as follows (all units g/L): K_2HPO_4 14; KH_2PO_4 6; $MgSO_4.7H_2O$ 0.2; trace mineral solution 2 mL; pH (at 25ºC) = 7±0.2. The trace mineral solution consisted of (all units g/L), EDTA 0.01; $ZnSO_4.7H_2O$ 0.0001; $CaCl_2.2H_2O$ 0.1; $MnCl_2.2H_2O$ 0.008; $FeCl_3.6H_2O$ 0.71; $(NH_4)_6Mo_7O_{24}$ 0.00011; $CuSO_4.5H_2O$ 0.0001; $CoCl_2.6H_2O$ 0.2. Glucose and acetate were used as carbon source. Ammonium chloride (NH_4Cl) and Potassium Nitrate (KNO_3) were used as nitrogen source. All such media were sterilized in a autoclave and the sterilized media were inoculated with 1% (v/v) of the isolate culture of P1 strain and incubated at 37ºC with shaking (100 rpm) for 24 hr divided into 12 hr anoxic and 12 hr aerobic. Anoxic condition was maintained by flashing CO_2 within the Erlenmeyer flask at the beginning of reaction. At the end of anoxic phase aeration tube was inserted inside the Erlenmeyer flask aseptically for supply of oxygen in subsequent aerobic phase.

At first, a preliminary 24 hr batch study was performed for studying the phosphate removal trend of the isolate P1 followed by a series of batch kinetics studies with different process parameters, to observe the potentiality of isolate P1 in simultaneous carbon oxidation and nutrient removal (N and P) removal efficiency. Aliquots were removed from each Erlenmeyer flask and growth was recorded by measuring OD_{600}. Next, the liquid was centrifuged at 10,000 rpm for 10 minutes and the supernatant was analyzed for pH, MLSS, MLVSS, SCOD, NH_4^+-N, NO_3^--N and phosphorous as per Standard Methods [29]. The pH of the solution was measured by a digital pH meter (Systronics, India make). NH_4^+-N and NO_3^- -N were estimated by respective ion selective electrodes in Orion ISE, meter. SCOD was analyzed by closed reflux method using dichromate digestion principle and HACH, USA digester. Dissolved oxygen (DO) was measured electrometrically by digital DO meter (Systronics, India make). Mixed liquor suspended solids (MLSS) and Mixed liquor volatile suspended solids (MLVSS) were measured by gravimetric method at temperature of 103-105℃ and 550±50℃ in muffle furnace, respectively. The soluble PO_4^{3-}-P of the sample was measured through the Vanadomolybdophosphoric Acid colorimetric method using UV/Visible spectrophotometer at 420 nm wavelength.

2.5.1 The effect of different types of organic carbon source on phosphorous removal by isolate P1

The effect of different types of organic carbon as substrate source on phosphorus removal was examined in a 24 hr duration batch experiment by using glucose and acetate as a carbon source in following experimental sets of combination.

Set-A (SCOD as Glucose =1000±50 mg/L, NH_4^+-N=50±10 mg/L, NO_3^--N=10±2mg/L, PO_4^{3-}-P=10±2 mg/L)

Set-B (SCOD as Glucose + Acetate=1000±50 mg/L, NH_4^+-N=50±10 mg/L, NO_3^--N=10±2mg/L, PO_4^{3-}-P=10±2mg/L)

2.5.2 Effect of Initial SCOD concentration on Phosphate Removal by isolate P1

The effect of initial SCOD level on phosphorus removal for different sets as described here under examined in a 24 hr batch experiment with the initial feed composition PO_4^{3-}-P=10±1 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L. The initial SCOD of Sets 1 to 4 was varied in the

range of 500±20 mg/L up to 1200±20 mg/L. The effect of initial SCOD level on phosphate removal performance was studied for following combination.

Set-1 (SCOD with acetate=500±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=10 ±1 mg/L)

Set-2 (SCOD with acetate=600±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=10±1 mg/L)

Set-3 (SCOD with acetate=800±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=10±1 mg/L)

Set-4 (SCOD with acetate=1200±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=10±1 mg/L)

2.5.3 Effect of initial PO_4^{3-}-P concentration on phosphate removal by isolate P1

The effect of initial PO_4^{3-}-P concentration on phosphorus removal was examined in a 24 hr batch study with an initial feed composition of SCOD=600±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2mg/L. The initial PO_4^{3-}-P concentration was varied from 5±1 to 20±1 mg/L in different combination of Sets from 5 to 8 as given below. The initial phosphate loading rate apparently induces a significant effect on PO_4^{3-}-P removals.

Set-5 (SCOD with acetate=600±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=5 ±1 mg/L)

Set-6 (SCOD with acetate= 600±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=10±1 mg/L)

Set-7 (SCOD with acetate= 600±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=15±1 mg/L)

Set-8 (SCOD with acetate=600±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=20±1 mg/L)

2.5.4 Combined carbon, nitrogen and phosphorous removal by isolate P1

To study the combined carbon, nitrogen and phosphorous removal kinetics, the isolate was cultivated batch wise in the basal medium. The experiment was carried out in two sets, where in each set was supplemented with distinct glucose and acetate (carbon source) concentration in presence of constant ammonium chloride (nitrogen source) and constant phosphate concentration. Kinetics study for simultaneous removal of organic carbon, NH_4^+-N, NO_3^--N and PO_4^{3-} -P has been undertaken for Set-9 and Set-10.

Set-9 (SCOD with acetate=600±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=5 ±1 mg/L)

Set-10 (SCOD with acetate=1000±20 mg/L, NH_4^+-N=50±5 mg/L, NO_3^--N=10±2 mg/L, PO_4^{3-}-P=10±1 mg/L)

3. RESULTS AND DISCUSSION

3.1 Identification of the Isolate P1 which Predominated in the Mixed Culture

3.1.1 Identification by growth on nutrient agar plate

A pure culture of dominant bacterial colony was obtained from the previously acclimatized mixed sludge by serial dilution technique (up to 10^{-9}) which subsequently grown on nutrient agar plates at 37°C. The image of the isolated pure culture in nutrient agar plate is shown in Fig. 1. It was observed that the culture seed secrets a greenish coloured pigment in the plate. However the growth of distinct colony was not visible from the plate which shows a diffused kind of growth of the bacterial strain P1. The green pigment and the diffused growth relates to *Pseudomonas sp.* generally, which was subsequently tested for confirmation of its identity by 16S rDNA study.

Fig. 1. Growth of isolate *Pseudomonas sp.* P1 on a nutrient agar plate

3.1.2 Identification by gram's staining

The isolated pure culture of the dominant bacteria from the acclimatized seed was identified through Gram staining, as the isolate P1 was found Gram negative. The microscopic image of the bacterial smear after staining has been given in Fig. 2. The image demonstrated that the cell retain the safranin stain only, thereby appear in pinkish colour indicates that the bacterium is Gram negative. The Fig. 2 also demonstrates that the bacterium is cocco bacillus shape resembling an intermediate between coccus (spherical) and bacillus (rod-shaped).

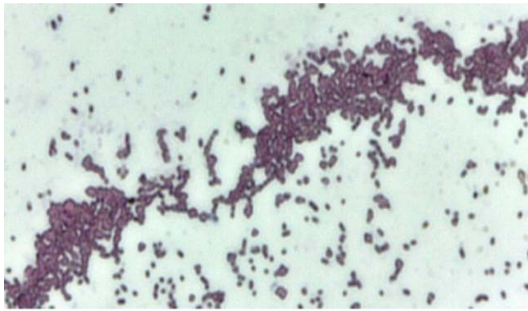

Fig. 2. Microscopic image of the isolate *Pseudomonas sp.* P1 after gram's staining

3.1.3 Identification by Sudan black staining

Sudan Black staining was also carried out as after the anoxic stage, the PHAs inside the cell of PAOs can be viewed only under the microscope by Sudan Black staining [30]. The microscopic image obtained after Sudan Black staining shown in Fig. 3. The PHA stored inside the cell got stained by Sudan Black dye and viewed as black spots inside the cells.

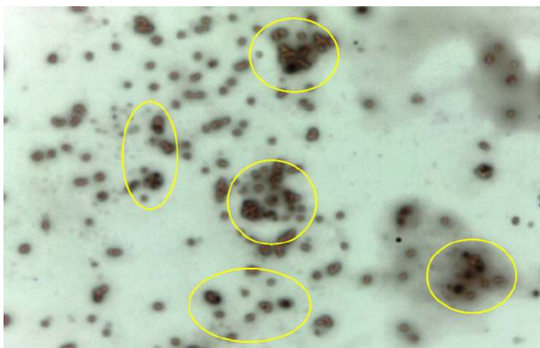

Fig. 3. Microscopic image of the isolate *Pseudomonas sp.* P1 after sudan black staining

3.2 Morphological and Physiological Characterization of Isolate P1

Morphological and physiological characteristics of the isolate P1 are listed in Table-1. In the nitrate reduction test, gas formation was observed in the Durham's tube indicating that the isolate P1 had the ability to convert nitrate to nitrite and ultimately to nitrogen gas. The isolate P1 was found to positive for both nitrate reductase and nitrite reductase. The isolate P1 utilized various organic compound such as D-Glucose, fructose, mannitol, glycerol, sodium citrate, sodium acetate, propionate, lactate, malate, ethanol, butyrate, propanol, butanol, benzoate, succinate, pyruvate, fumarate as their carbon source and energy requirement. The isolate P1 utilized various nitrogen compound and amino acid such as ammonium acetate, ammonium nitrate, potassium nitrate, ammonium chloride, ammonium sulphate, sodium nitrate, peptone, alanine, lysine, leucine, histidine, asparagines, aspartate, proline, arginine as their nitrogen source. The isolate P1 utilized various phosphate compounds such as ammonium-di-hydrogen phosphate, di-ammonium hydrogen phosphate, potassium-di-hydrogen phosphate, di-potassium hydrogen phosphate for their cell growth and store them as a polyphosphate. This enrichment of the isolated bacteria (*Pseudomonas sp.* P1) cell containing a high concentration of polyphosphate leads to the establishment of biological phosphorus removal (BPR). The morphological and physiological characteristics of isolate P1 identified as gram negative coccobacillus with effective growth between 25-42ºC in 24 hr. The Scanning Electron Microscope (SEM) view of the isolate P1 is shown in Fig. 4.

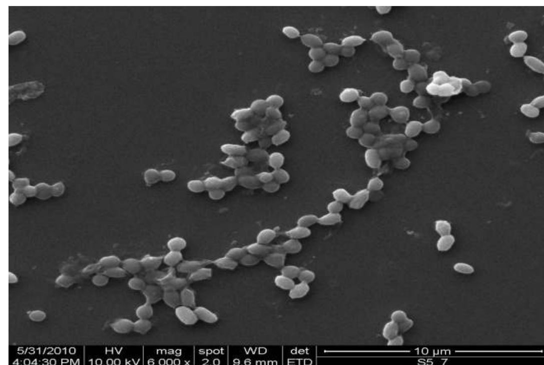

Fig. 4. SEM picture of isolate *Pseudomonas sp.* P1 under scanning electron microscope

Table 1. Morphological and physiological characteristics of *Pseudomonas sp.* P1

Morphology	
Cell shape	Coccobacillus, individual cells or in clusters
Motility	Flagella unipolar
Gram-staining	Negative
Colony morphology (nutrient agar)	Diffused colony with greenish colored pigment
Growth conditions	
Temperature range	Good growth at 25–42ºC
pH range	6.5–8.5
Relation to oxygen	Aerobic and anaerobic nitrate respiration
Growth in the presence of 6.5 % NaCl	Positive
Physiological characteristics	
Negative	Acid production from sucrose, lactose, xylose, sorbitol, and arabinose, urease, DNA hydrolysis, methyl-red test, levan formation, Indole test, Voges Proskauer test, *o*-nitrophenyl-β-galactoside, lecithinase
Positive	Acid production from glucose, fructose and ribose,indophenols oxidase,nitrate reduction, gelatin liquefaction, lipase, citrate utilization, catalase
Utilization of organic compounds as sole nutrient	
Negative	Sucrose, lactose, xylose, sorbitol, L-rhamnose, cellobios, maltose , L-Arabinos, trehalose, inulin, starch, salicin, methanol, D-mannose, ethylene glycol, phenol, tartrate
Positive	D-Glucose, fructose, mannitol, glycerol, sodium citrate, sodium acetate, propionate, lactate, malate, ethanol, butyrate, propanol, butanol, benzoate, succinate, pyruvate, fumarate
Sole nitrogen source utilization test	
Negative	Urea, cobaltous nitrate, threonine
Positive	Ammonium acetate, ammonium nitrate, potassium nitrate, ammonium chloride, ammonium sulphate, sodium nitrate, peptone, alanine, lysine, leucine, histidine, asparagines, aspartate, praline, arginine
Phosphorous utilization test	
Positive	Ammonium-di-hydrogen phosphate, di-ammonium hydrogen phosphate, potassium-di-hydrogen phosphate, di-potassium hydrogen phosphate

3.3 16S rDNA Gene Sequence and Phylogenetic Analysis of Isolate P1

On the basis of nucleotide homology and phylogenetic analysis with the 1393 bp 16S rDNA gene sequence of the isolated strain P1, it was found that the isolate *Pseudomonas sp.*P1 strain (Genbank accession number KM982695) have a significant similarity with *Pseudomonas aeruginosa*. The BLAST alignment result of the sequence isolate strain P1 showed 99% similarity with the *Pseudomonas aeruginosa.* After undergoing the Multiple Sequence Alignment of the reported sample DNA sequence (isolate strain P1) with the other sequences found in the BLAST study, a Phylogram (Fig. 5) was drawn showing its position along with the species *Pseudomonas aeruginosa*. The multiple sequence alignment along with the sample sequence of isolate P1 forms a single cluster. But the distance of isolate P1 is slightly greater than the other sequence showing a marginal difference from the reported *Pseudomonas aeruginosa* species listed in NCBI. Strain P1 emerged as a distinctive phylogenetic line from the cluster containing the other type strains of *Pseudomonas* species shown in Fig. 5.

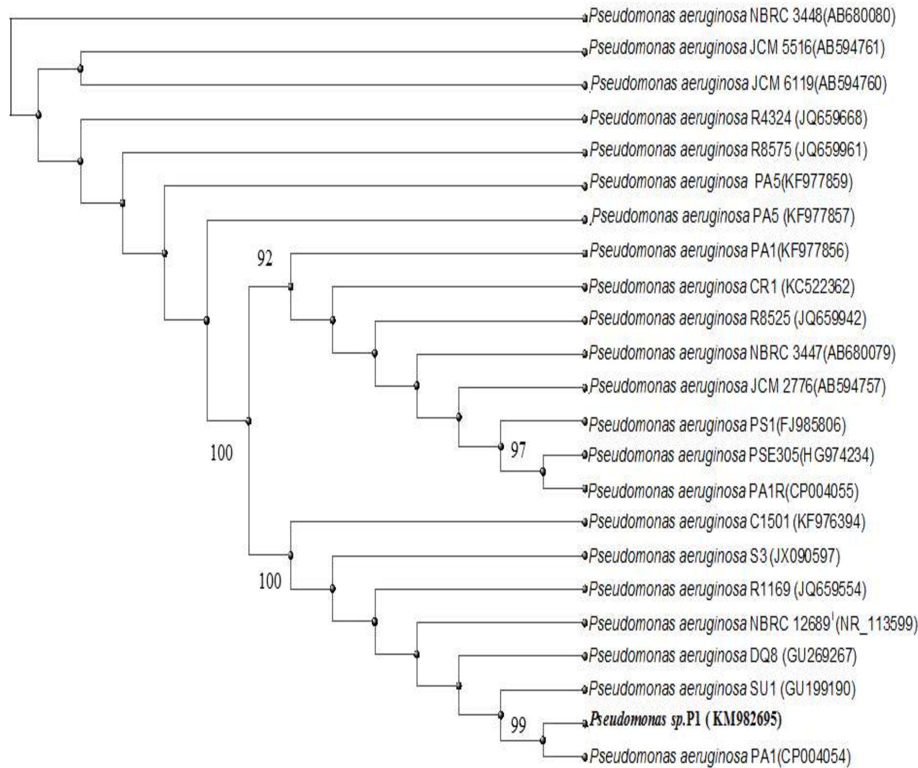

Fig. 5. Unrooted phylogenetic tree based on 16S rDNA gene sequences obtained by the Neighbor-Joining (NJ) method showing the position of strain *Pseudomonas sp.*P1among its phylogenetic neighbors. NCBI accession numbers are provided in parentheses

3.4 Effect of Different Types of Organic Carbon Source on Phosphorous Removal by Isolate P1

Two sets (Set-A and Set-B) of batch study were carried out to examine the effect of the presence of a specific type of organic carbon source during phosphate assimilation. Dextrose with sodium acetate (CH_3COONa) in the feed has been chosen for conducting necessary test, on phosphate uptake by the acclimatized culture. The initial PO_4^{3-}-P concentration was around 10±2 mg/L for both the sets. The phosphorous removal for different organic carbon source in the reactor has been plotted in Fig. 6. In the first set (Set-A), organic carbon was added in the form of dextrose ($C_6H_{12}O_6$) only, targeting an initial SCOD concentration of 1000±50 mg/L. The test result exhibited a marginal phosphate removal with a net removal efficiency of 39.01% only. The SCOD removal efficiency was obtained as 78.29% after 24 hr of overall react period. The 39.01% reduction of phosphorus might be attributed to metabolic phosphorus requirement of bacterial cell for cell synthesis. Considering a low phosphorus release, being attributed during acetate limiting conditions, in the second phase of the study a sodium acetate equivalency of approximately 500±25 mg SCOD/L along with glucose, having an equivalent SCOD level of 500±25 mg/L, was added to the feed solution in the second set (Set-B) to have an overall SCOD level of 1000±50 mg/L for enhancing the phosphate release in the anoxic phase. A marked improvement in net phosphate removal efficiency was elevated from 39.01 to 85.75% with a higher SCOD removal efficiency of 86.71% also. The results corroborate the earlier observation as made by Tasli et al. [31] to this effect that nature and type of organic substrate available in the solution plays a key role for effective phosphorus removal which selectively requires the existence of short-chain fatty acids viz. acetic acid or acetate. Akin and Ugurlu [6] also found that the phosphate removal using acetate as carbon source along with glucose was found to be more effective (68.7%) compare to glucose feeding (37.8%) as carbon source.

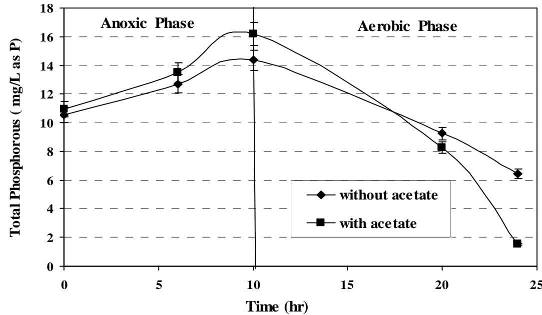

Fig. 6. Effect of different organic carbon source on phosphorous removal

3.5 Effect of Initial SCOD of the Feed on Phosphate Removal by Isolate P1

The effect of initial SCOD level on phosphorus removal of different experimental sets of varying initial SCOD concentration were examined in a 24 hr batch study with the initial feed composition of PO_4^{3-}-P=10 mg/L, NH_4^+-N=50 mg/L, NO_3^--N=10 mg/L. The initial SCOD concentrations of different experimental sets were varied in the range of 500 to 1200 mg/L. Results on time-course study with respect to different initial SCOD at constant initial PO_4^{3-}-P concentration of around 10 mg/L has been plotted in Fig. 7. The maximum SCOD removal of 96.59% occurs at initial SCOD concentration of 617 mg/L. The phosphorous removal (%) with time for different initial SCOD value in the reactor has been exhibited in Fig. 8 which shows that the phosphorous removal (%) was lower (56.61%) at lower initial SCOD concentration such as 500±20 mg/L in comparison of higher initial SCOD concentration such as 600±20, 800±20 and 1200±20 mg/L. However, the phosphorous removal (82.25%), which reaches its peak at initial SCOD of 600±20 mg/L, marginally decreased in presence of higher SCOD concentration of 800±20 and 1200±20 mg/L.

3.6 Effect of Initial Phosphorous Concentration on Phosphate Removal by Isolate P1

Phosphorous removal efficiency also depends on initial phosphate loading to the reactor for which the effect of initial phosphorous concentration on phosphorus removal percentage was further examined with an initial feed composition of SCOD = 600±20 mg/L, NH_4^+-N = 50±5 mg/L, NO_3^--N = 10±2 mg/L in batch mode. The initial PO_4^{3-}-P concentration was varied progressively from 5±1 to 20±1 mg/L in different sets of experiment. Time-concentration study with respect to different initial PO_4^{3-}-P concentration for the isolate *Pseudomonas sp.*P1 at constant initial SCOD concentration of 600±20 mg/L are plotted in Fig. 9 that clearly demonstrates that up to a value of 10.6 mg/L of initial PO_4^{3-}-P concentration, the removal efficiency increases after which the uptake is descended. For the initial PO_4^{3-}-P concentration of 10.6 mg/L, the maximum phosphorous removal efficiency (89.15%) was obtained after 24 hr. The removal efficiency dropped down slightly to 78.43% when initial PO_4^{3-}-P concentration was increased to 19.20 mg/L.

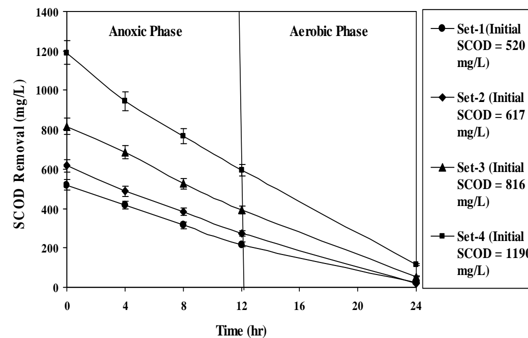

Fig. 7. Time profile of SCOD removal by the isolate *Pseudomonas sp.*P1

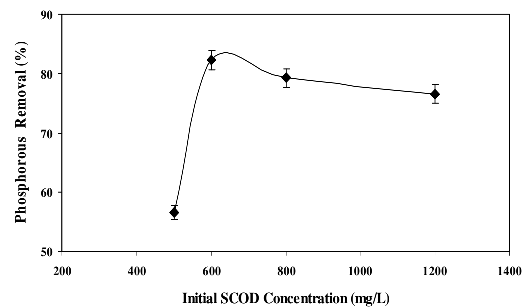

Fig. 8. Phosphorus removal (%) under different initial SCOD concentration by the isolate *Pseudomonas sp.*P1

3.7 Simultaneous Carbon-Oxidation and Nutrient (N and P) Removal by Isolate *Pseudomonas sp.* P1

As the isolate *Pseudomonas sp.*P1 showed both denitrifying ability along with phosphate removal capacity with carbon utilization, a combined CNP (Carbon, Nitrogen and Phosphorus) removal

performance study was hence carried out in the same batch reactor. Two sets of batch studies viz. Set-9 and Set-10 were carried out with varying SCOD concentration. In Set-9, the targeted initial feed composition were SCOD=600±20 mg/L, PO_4^{3-}-P=10±1 mg/L, NO_3^--N=10±2 mg/L and NH_4^+-N=50±5 mg/L. The results on time-concentration study for SCOD, PO_4^{3-}-P, NO_3^--N and NH_4^+-N removal are plotted in Fig. 10 which showed that the nitrate level descended 90.26% after 12 hr of anoxic period. There was no notable change in the NH_4^+-N level in the anoxic period. The phosphate level was increased slightly which exhibits accumulation of PHA by the isolate *Pseudomonas sp.*P1. The SCOD level also decreased reasonably indicating utilization of organic carbon source by isolate *Pseudomonas sp.*P1 in anoxic phase. The next 12 hr of aerobic period yields a total SCOD removal of 94.30%, total phosphorous removal of 87.98% and NH_4^+-N removal of 81.38%. The nitrate level increased marginally in the aerobic phase owing to the possible conversion of NH_4^+-N into NO_3^--N, which decreased the overall nitrate removal into 72.78%. A part of ammonia was used by the bacteria for cell synthesis also.

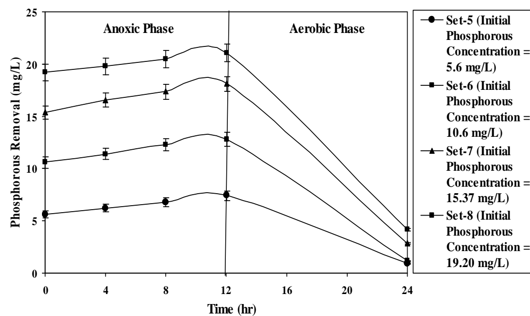

Fig. 9. Time profile of phosphorous removal by the isolate *Pseudomonas sp.*P1

Set-10 was carried out at a higher organic loading to observe the removal performance at higher organic loads. The initial SCOD level was increased to around 1000±20 mg/L, keeping the concentration of other nutrient levels are same. The kinetic result with time for SCOD, PO_4^{3-}-P, NO_3^--N and NH_4^+-N removal is shown in Fig. 11. From Fig. 11, it was observed that the nitrate removal efficiency in Set-10 (71.36%) did not deviate much from that found in Set-9 (72.78%). So, it could be concluded from the results obtained in Set-9 and Set-10 that the nitrate removal performance did not depend on initial SCOD concentration and isolate *Pseudomonas sp.*P1 have denitrifying activity. The phosphate removal efficiency reduced to 80.79% in Set-10 from 87.98% in Set-9 due to higher initial SCOD available to biomass inside the reactor which also increases the SCOD: P ratio is around 92.27 in Set-10 from 60.29 in Set-9. The most important observation for the NH_4^+-N removal efficiency in Set-10 (80.17%), which nearly equal to the NH_4^+-N removal in Set-9 (81.38%), indicating the isolate *Pseudomonas sp.*P1 have nitrifying activity.

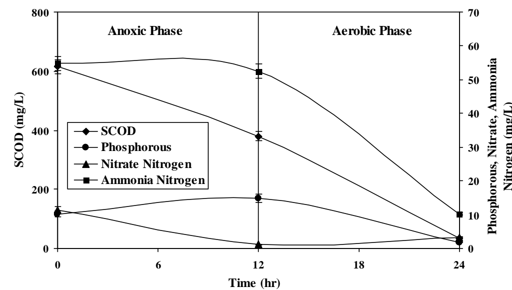

Fig. 10. Simultaneous removal profile of organic carbon and nutrients at initial SCOD = 600±20 mg/L by the isolate *Pseudomonas sp.* P1

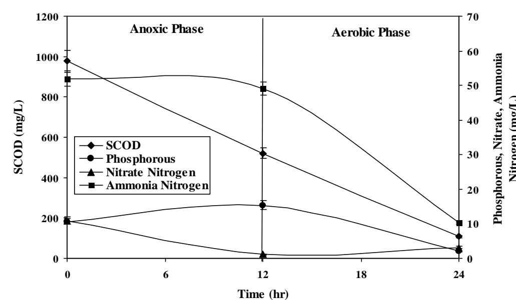

Fig. 11. Simultaneous removal profile of organic carbon and nutrients at initial SCOD = 1000±20 mg/L by the isolate *Pseudomonas sp.* P1

4. CONCLUSION

Biological phosphorus removal (BPR) accomplished in anoxic environment followed by aerobic environment in sequential manner which is successfully observed in one single batch reactor. The batch performance studies exhibited that the phosphate removal is sensitive to organic source (acetate) in the feed and the amount of biomass supplied. The bacterium isolated from slaughterhouse wastewater and identified as *Pseudomonas sp.* P1 successfully

stabilized SCOD (soluble organics) as well as ammonium nitrogen and nitrate nitrogen. The bacterium was capable of utilizing phosphate aerobically in presence of NH_4^+-N. The bacterium *Pseudomonas sp.* P1 shows the 99% similarity with *Pseudomonas aeruginosa.* Further taxonomic investigations are warranted to establish the strain as a new species and to be tolerant in real life slaughterhouse wastewater.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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