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Variation of extracellular polymeric substances (EPS) and specific resistance to filtration in sludge granulation process to the change of influent organic loading rate

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ABSTRACT

Four sequencing batch reactors (SBRs) were operated to evaluate the effects of changing the influent chemical oxygen demand (COD) loading rate (F/M ratio) on various components of EPS and their relationship with other physicochemical characteristics of granular sludges in the SBR system. F/M ratio in R-1, R-2, R-3, and R-4 was 0.11, 0.21, 0.32, and 0.43 kg COD/kg mixed liquor suspended solids (MLSS)·d, respectively. The sludge fed with higher F/M ratio enhanced the granulation process and produced more polysaccharides (PS) and proteins (PN) than the sludge run with lower COD loading rate while humic substances and uronic acids did not show any profound difference in their contents. The total EPS contents in the granular sludges were higher at the beginning of experiment as compared to their values noted at the end of studies. The granular sludge settleability was positively correlated with the F/M ratio and EPS contents. Specific resistance values at the end of studies were 2.37×10^8, 1.38×10^8, 1.86×10^8, and $9.32 \times 10^8\,s^2/g$ in R-1, R-2, R-3, and R-4, respectively, to that of seed sludge ($6.80 \times 10^9\,s^2/g$). The higher values of specific resistance in R-4 were due to raised contents of PS compared to other reactors. EPS particularly PS were found to be positively correlated with settleability and negatively with dewatering characteristics of sludges.

Keywords: Granular sludge; Food/microorganisms ratio; Extracellular polymeric substances; Dewaterability; Settleability

1. Introduction

Extracellular polymeric substances (EPS) are produced by the metabolic activities and cell lysis of microorganisms [1]; they play very important role in the biogranulation process, flocculation and settleability, dewatering of granular sludge, and other physicochemical characteristics of aerobic granules [2,3]. A variety of functional groups such as hydroxyl, carboxyl, phenolic, and phosphoric are present in the EPS molecules, which are significant for binding the particles during granulation process through

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hydrophobic interactions, hydrogen bonding, and ionic interactions. EPS is a sticky material and therefore facilitate the cell-to-cell adhesion to unite the microbial biomass which is very vital for the start of granulation process [4].

Aerobic granulation process is a novel environmental technique and aerobic granules have excellent settleability, smooth and round shape, high biomass retention and tolerance to toxicity in removing nitrogen, phosphorous, and organics etc. from the wastewater [5,6]. Liu and Tay [7] proposed a model to describe the mechanism of aerobic granulation process in which they highlighted various important physical and chemical factors responsible for the granulation but the key point among those was EPS. Some studies suggested that the type and physical characteristics of individual biopolymers (EPS) are very important along with their total amount in the bioflocculation process [8].

The chemical composition of EPS is very complex, mainly includes polymers like carbohydrates, proteins (PN), humic substances, uranic acids, and phospholipids [9–11]. The composition and nature of EPS produced strongly depends upon the organic loading rate and substrates used as food in the wastewater [12,13]. But, the effects of EPS produced on other physicochemical characteristics of granular sludges at the same time have not been significantly studied. Wang et al. [14] studied the EPS distribution in aerobic granules and found that EPS contents in inner side of granules are much higher as compared to the outer side. Protein is considered to be the main polymeric substance responsible to hold the granules together and is present in the inner layer, while carbohydrates concentrate on the outer surface of aerobic granules [15].

The role of EPS in characterizing the dewaterability (specific resistance to filtration) and settleability of sludges is very complex and contradictory results have been reported by different researchers and no clear relationship has been established so far. Some researchers found that EPS molecules retain sufficient water due to hydrogen bonding, therefore higher contents of EPS in the sludge weaken their dewaterabilities [16,17], while others have evaluated that an increase in EPS concentration had a positive effect on the dewatering characteristics of sludges and it improved as the EPS augmented in its contents [2,18]. The specific resistance to filtration is related to hydrophilic and hydrophobic characteristics of granular sludge, the more hydrophobic is the sludge the higher is its dewaterability and vice versa. The higher EPS contents are positively correlated with the settleability of sludges [19], while others noted that settleability decreased with an increase in EPS contents [20,21]. Moreover, these studies were mainly carried out on activated sludges at constant influent loading rates and less work has done on granular sludges, particularly at varied F/M ratio for describing these sludge characteristics.

Due to the complex nature of biochemical processes involved, the more extensive studies are required to understand the relationship of EPS with specific resistance to filtration (dewaterability) and other physicochemical characteristics of granular sludges, particularly at different influent organic loading rates because it yields the different compositions of EPS during the granulation process. Therefore, the main purposes of this study were to find out the effects of changing organic loading rates on the production and variation of EPS during granulation process in SBR system as well as to elucidate the role of individual EPS components in determining the granular sludge dewaterability (specific resistance to filtration) and settleability characteristics for the wastewater treatment.

2. Materials and methods

2.1. Reactor parameters and operation

In the present study, four geometrically identical column reactors named as R-1, R-2, R-3, and R-4 of 3.14 L (40 cm height and 10 cm inner diameter) capacity each, were operated as the sequential batch reactors. The working volume of each reactor was 3.0 L and influent exchangeable volume was 1.6 L. The influent was added from the top by pumps and effluent discharged from the specific point at each reactor (above the sludge blanket level) by using solenoid valves. The air was supplied from the bottom of reactors and controlled by gas flow regulators. All the operations were automatically controlled by timers attached to each of them. The schematic diagram of reactor is given in Fig. 1. The temperature of reactor room was maintained at 25°C by automatic thermo controller. All the reactors were operated under similar conditions except the influent chemical oxygen demand (COD) loading rate in terms of glucose. Batch cycle time of each reactor was 6 h (four cycles/day) and each cycle consisted of 10 min feeding, 310 min of aeration, 15–30 min of settling, and 10 min draining. The settling time decreased from 30 to 15 min with the increase in sludge settleability. The operational period of experiment was one month. All the important parameters maintained in the experiment are shown in Table 1.

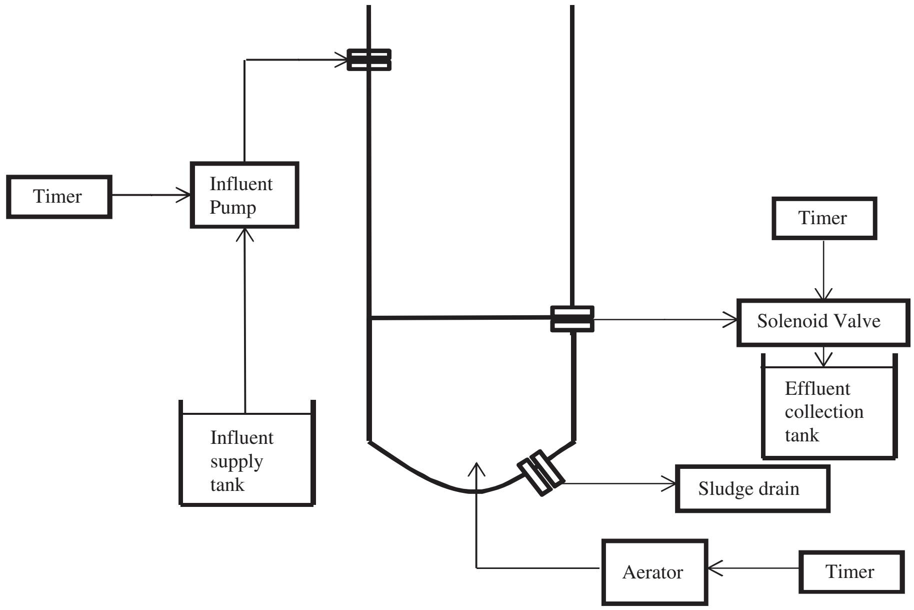


Fig. 1. A schematic diagram of reactor.

Table 1
Operating conditions of R-1, R-2, R-3, and R-4.

Reactors	R-1	R-2	R-3	R-4
MLSS (mg/L)	4,000	4,000	4,000	4,000
Initial SVI_{30} ml/g (seed sludge)	180	180	180	180
Influent COD concentration (mg/L)	200	400	600	800
F/M ratio (kg COD/kg MLSS·d)	0.11	0.21	0.32	0.43
Operational pH of synthetic waste water	7.0	7.0	7.0	7.0
Temperature (℃)	25	25	25	25
Feeding time (min)/cycle	10	10	10	10
Aeration time (min)/cycle	310	310	310	310
Settling time (min)/cycle	30–15	30–15	30–15	30–15
Drainage time (min)/cycle	10	10	10	10

2.2. Seed sludge and composition of synthetic wastewater

The aerobic activated sludge was taken from local municipal wastewater treatment plant in llsen, South Korea as seed sludge and the initial mixed liquor suspended solids (MLSS) concentration of all reactors was adjusted to 4,000 mg/L. The sludge was dark in color and its sludge volume index (SVI_{30}) was 180 mL/g at the beginning. Reactors were operated at different F/M ratio i.e. 0.11, 0.21, 0.32, and 0.43 kg COD/kg MLSS·d in R-1, R-2, R-3, and R-4, respectively throughout the studies. The initial influent COD loading rate was adjusted at 200, 400, 600, and 800 mg/L in R-1, R-2, R-3, and R-4, respectively by adding 190, 380, 570, and 760 mg/L glucose in the wastewater of these reactors. The composition of other constituents in waste water of all reactors was same and is given as follows; NH_4Cl 87 mg/L, KH_2PO_4 25 mg/l, K_2HPO_4 50 mg/L, Na_2CO_3 60 mg/L, $CaCl_2$ 5 mg/L, $MgSO_4$ 2 mg/L, and trace element solution 1 mL/L. The composition of trace element solution was, $FeCl_3$ 20 mg/L, $CuSO_4$ 50 mg/L, $MnSO_4{\cdot}H_2O$ 50 mg/L, $CoCl_2{\cdot}6H_2O$ 50 mg/L, KCl 8 mg/L, $AlCl_3$ 15 mg/L, $ZnSO_4{\cdot}7H_2O$ 30 mg/L, H_3BO_3 40 mg/L, and $(NH_4)_6MoO_{24}{\cdot}4H_2O$ 30 mg/L [22]. The pH of the wastewater was maintained at 7.0 by phosphate buffer.

MLSS concentration in R-1 decreased continuously in the present studies due to its operation at comparatively low F/M ratio (0.11 kg COD/kg MLSS·d). Therefore, its influent COD loading rate decreased from 200 to 155 mg/L with the progress of experiment to ensure stable F/M ratio. Similarly, the MLSS concentration of all other reactors checked daily and initial influent COD loading rates adjusted accordingly to have an even F/M ratio throughout the experimental investigations.

2.3. Granular sludge and effluent samples analysis

To ensure the stable operation in all SBRs various parameters like pH, COD, mixed liquor suspended solids, sludge volume index (SVI_{30}), NH_4–N and PO_4–P of granular sludge, and effluent samples were monitored on a regular basis in accordance with the standard methods [23].

2.4. Microscopic studies and particle size analysis of sludge

The structural observations of microbial organisms and morphology of aerobic granules in SBRs were studied by using biological compound microscope MX-4300H (Meiji Techno Japan). All studies were conducted at plan objective magnification of 4X and instrument was equipped with the computer. The particle size distribution of granular sludge was performed by using optic laser diffraction HELOS/KF-MAGIX (Sympa Quixel Germany) particle size analyzer which had a working range of 0.1–3,500 μm.

2.5. Specific resistance

The granular sludge dewaterability in terms of specific resistance to filtration was measured by using Buchner Funnel apparatus. The sludge sample of 80 mL was added in the Buchner Funnel, the filtration was conducted by using Whatman No. 42 filter paper at a constant vacuum of $-80 \times 10^4\,g/cm^2$ and time of filtration to the volume of filtrate collected was noted. From the data obtained, specific resistance was measured by using the integrated forms of Darcy's equation for filtration which are as follows [24]:

$$t/V = [\mu rc/2PA^2.V + \mu Rm/PA] \quad (1)$$

$$r = 2PA^2 b/\mu c \quad (2)$$

where r is the specific resistance (s^2/g), P is the applied vacuum (g/cm^2), A is the area of filter paper (cm^2), b is the slope ($\mu rc/2PA^2$) of line from Eq. (1) which is obtained by plotting the ratio of time of filtration to volume of filtrate (t/V) vs. volume of filtrate (V) obtained from Buchner Funnel apparatus, μ is the filtrate viscosity (g/cm s) and c is the solids per unit volume of filtrate obtained from initial solid contents of influent sludge and final solid contents of cake sludge.

2.6. EPS of granular sludge and effluent samples

2.6.1. Extraction of EPS

The boundary EPS of sludge samples were extracted using cation exchange resin (CER) method [10]. The CER was in sodium form, Dowex 50 × 8, 20–25 mesh from Fluka (44445).The extraction was carried out at 4°C and 300 rpm for 3 h in shaking incubator to facilitate much rapid withdrawal of EPS from granular sludge samples. The extracted EPS were first centrifuged at 25,000 × g by using GYROZEN 1580 MGR centrifuge machine at 4°C for 15 min, and secondly at 15,000 × g at 4°C for 15 min. The extracted EPS were filtered through 0.20 μm filters and filtrate was stored at −20°C until the required determinations were made [25]. The CER is a better technique to extract EPS due to its low cell lysis and exopolymer disruptions [10]. The samples of effluent from all the four reactors were also collected on daily basis and filtered through 0.20 μm filters to remove any suspended solids and filtrate stored at −20°C as discussed earlier for the EPS analysis, these were called as soluble EPS in present studies.

2.6.2. Spectrophotometric analysis of EPS

Both types of EPS were analyzed by using HACH DR/4000 Spectrophotometer. The carbohydrate contents of EPS were determined by phenol–sulphuric acid method of Dubois et al. [26] using glucose as the standard solution. PN and humic substances were determined by modified Lowery method, Frolund et al. [27] using bovine serum albumin (Sigma–Aldrich) and humic acid (Aldrich) as the respective standards. m-hydroxydiphenyl sulphuric acid method of Blumenkrantz and Asboe-Hansen [28] was used for the determination of uronic acid. Glucuronic acid (Sigma) was used as the standard solution. In each determination blanks were also run as control samples by adding respective extracting reagents and following the same standard procedures but omitting the samples. Finally, the absorptions were converted to concentrations of each constituent of EPS and also used the dilution factors where involved.

3. Results and discussion

3.1. Mixed liquor suspended solids (MLSS) concentration during granulation process

MLSS concentrations of all reactors were measured daily and influent COD loading rates were adjusted on regular basis from their initial fixed values of 200, 400, 600, and 800 mg COD/L in R-1 R-2, R-3, and R-4, respectively depending upon decrease or increase in their MLSS concentrations to ensure the constant F/M in all the reactors throughout the studies. A decrease in MLSS concentrations of all SBRs around 3,400 mg/L from their initial value of 4,000 mg/L was noted in the first three days of aerobic sludge granulation process indicating the lack of active microbial population in the seed sludge. Therefore, the COD loading rate in this period was adjusted around 175, 335, 510, and 685 mg/L in R-1 R-2, R-3, and R-4, respectively to have the same initial fixed F/M ratio of 0.11, 0.21, 0.32, and 0.43 in R-1–R-4, respectively. After this, F/M ratio displayed a profound effect on the MLSS concentration and a rapid increase in R-3 and R-4 due to high organic loading rate was noted, while R-2 had a less rise in it indicating a low growth of micro-organisms in this reactor. The extra sludge discharged daily from these reactors for comparable studies. On the other hand MLSS of R-1 decreased continuously and this trend was observed during the whole period of experiment. So, its COD loading rate decreased accordingly to result in a uniform F/M ratio. The variation in MLSS with time in R-1, R-2, R-3, and R-4 is shown in Fig. 2.

3.2. Role of F/M ratio and sludge EPS in aerobic granules formation

The seed sludge had SVI_{30} value of 180 mL/g with average floc size of 50–75 μm and was mixed well before inoculated into the reactors. The microscopic examination on day seven showed that sludge particles started to join each other to form biological aggregates in R-3 and R-4, while this trend was not very obvious in R-1 and R-2. The particle size analysis of samples from R-1, R-2, R-3, and R-4 at the same time revealed that the average particle size in these reactors was around 150, 180, 240, and 300 μm, respectively. The high growth rate of microbes in R-3 and R-4 as indicated by their MLSS concentrations caused a rapid increase in floc size in these reactors while it was less in R-1 and R-2 due to their operation at comparatively low F/M ratio. The difference in EPS concentrations of sludges in all the four reactors was also responsible for varied granulation process in the SBRs. EPS contents in terms of polysaccharides (PS) and PN were very high in R-3 and R-4 than R-1 and R-2 and measured as 88.41, 100.99, 139.47, and 159.30 mg/g SS in R-1, R-2, R-3, and R-4, respectively on day seven of experiment. The aerobic granulation is favored by an increase in polysaccharide and protein contents of EPS [3,4].

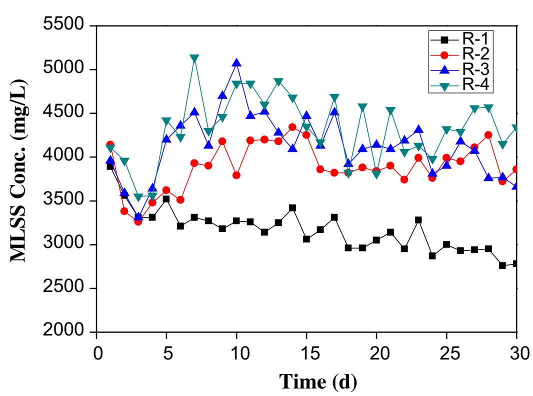


Fig. 2. MLSS concentration (mg/L) vs. time (d) of R-1, R-2, R-3, and R-4 in SBR granulation process.

The concentration of EPS in R-3 and R-4 (operated at high COD loading rate) increased rapidly at the start and it enabled the microbial cell interaction through polymeric network which helped to start the granulation process by increasing the floc size. R-1 and R-2 operated at low F/M ratio, produced less PS and PN, therefore the floc size was much smaller in these reactors. The small granules first appeared after 14 and 10 days in R-3 and R-4, respectively. The granulation process was much slower in R-1 and R-2 and it took about 21 and 18 days, respectively.

The morphology and structure of granules in reactors were different from each other. The granules of R-4 were bigger in size and had yellow color but towards the end of experiment, they started to scatter and converted into loose structure granules. It was further supported by its SVI values as it increased from 33.03 mL/g on day 21 to 44.34 mL/g on day 30. The average granule size in R-4 at the end of studies was around 1,150 μm. On the other hand the aerobic granules of R-2 and R-3 were compact in structure and at the end of experiment, they had no much difference in size with each other and average granule size was 950 μm in both the reactors while R-1 granule size was 700 μm. These results showed that high organic loading rate accelerated granulation process in R-4 and granules were much bigger in size as compared to the sludge fed with low F/M ratio in other rectors but compacted granules were obtained in R-2 and R-3. The microscopic observations at the end of experiment also revealed that particles in R-4 had scattered structure and those of other three reactors were very compact as shown in Fig. 3. A very useful correlation between granulation process and SVI_{30} of all rectors was found and it was noted that when SVI reached around 60 mL/g, granulation process was augmented and particles increased in their size rapidly.

3.3. Influence of F/M ratio on the sludge volume index (SVI_{30}) of granular sludges

The variation in SVI of reactors with time is shown in Fig. 4. The changes in the sludge's settleability in terms of SVI_{30} were measured during the granulation process. Initially for a period of three days, an increase in SVI_{30} values of all the four reactors around 200 mL/g were noted to that of inoculated seed sludge (180 mL/g). This poor compression of sludge was due to the appearance of filamentous bacteria in the SBRs as evidenced by the microscopic visuals at that time. Afterwards, the settleability of sludge improved significantly due to disappearance of these filamentous structures and SVI of R-4 on day seven was recorded as 64.20 mL/g. The SVI of R-2 and R-3 was almost double than R-4 and had values 125.36 and 119.95 mL/g, respectively while that of R-1 was 164.45 mL/g indicating the low settleability of sludge in this reactor. The difference in settling behavior of sludges was due to variation in the organic loading rate. The high COD loading rate in R-4 enhanced the production of microbes and EPS which helped to increase their particle size due to their adhesions with each other and as a result sharp decrease in SVI values was observed as compared to other reactors. The larger and denser particles settled quickly and decrease in SVI values was noted. The increase of EPS contents particularly PN had positive impact on the settleability of sludges in our studies. EPS contents at this stage were around 138, 160, 195, and 215 mg/g SS in R-1, R-2, R-3, and R-4, respectively and SVI values decreased with the increase of EPS contents. These results were in contradiction to the findings of [20,21] who observed that settling characteristics of sludge decreased with the increase in EPS contents but showed good agreement with the results of [19,29].

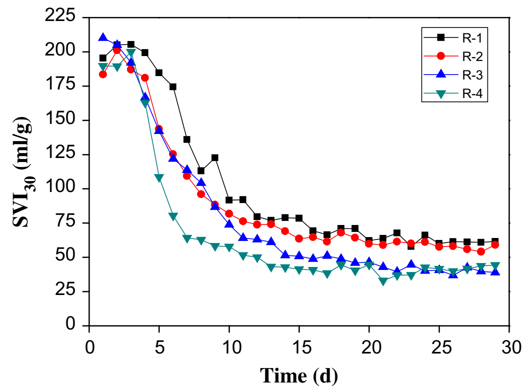
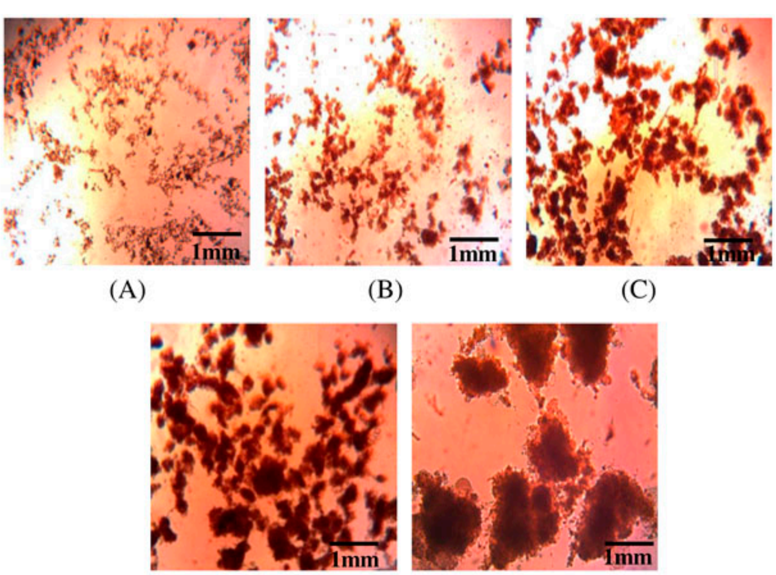


Fig. 4. Changes in SVI_{30} vs. time (d) in R-1, R-2, R-3, and R-4 during granulation process.



(A) (B) (C)

(D) (E)

Fig. 3. Microscopic pictures of (A) initial sludge, (B) R-1, (C) R-2, (D) R-3, and (E) R-4 after one month at 4× magnification.

The SVI of all reactors decreased continuously throughout the experimental investigation but at a slower pace compared to the first week of process. R-4 showed best settleability, when its SVI was 33.03 mL/g on day 21. Hereafter 21 days its SVI started to increase and observed 44.34 mL/g at the end of studies. As discussed previously, the particles in R-4 at the end of experiment had loose structures due to continued high loading rate which resulted in the deterioration of sludge settleability. The lowest value of SVI in R-3 was 40.20 mL/g on day 25 and no significant fluctuation observed in its settleability till the completion of experiment. R-1 and R-2 showed almost similar trend in the settling behavior of sludge in the second phase of studies and both had SVI values around 60 mL/g on day 23 and remained unchanged till end. It was important to note that R-4 had SVI value around 60 mL/g at the end of first week while R-1, R-2, and R-3 gained similar SVI on 23, 22, and 14th day of experiment, respectively. These results indicated that high organic loading rate showed a positive correlation with settleability of sludges by decreasing their SVI values and increasing EPS contents.

3.4. Extracellular polymeric substances in SBRs

3.4.1. Variation of sludge EPS with different F/M ratio in granulation process

The sludge EPS concentrations of all the four reactors showed an increasing trend at the initial stage of granulation to that of inoculated seed sludge (134.60 mg/g SS) unlike [30] who noted that EPS were decreased at the early stage of granulation process. The total EPS of R-3 and R-4 increased continuously and reached the maximum value of 197.17 and 220.87 mg/g SS, respectively after five days while those of R-1 and R-2 were 146.41 and 159.31 mg/g SS, respectively at the same time. There was an overall decreasing trend in the EPS contents of granular sludges with the progress of studies and matured granules at the end of studies had EPS 113.10, 121.10, 138.68, and 150.60 mg/g SS in R-1, R-2, R-3, and R-4,

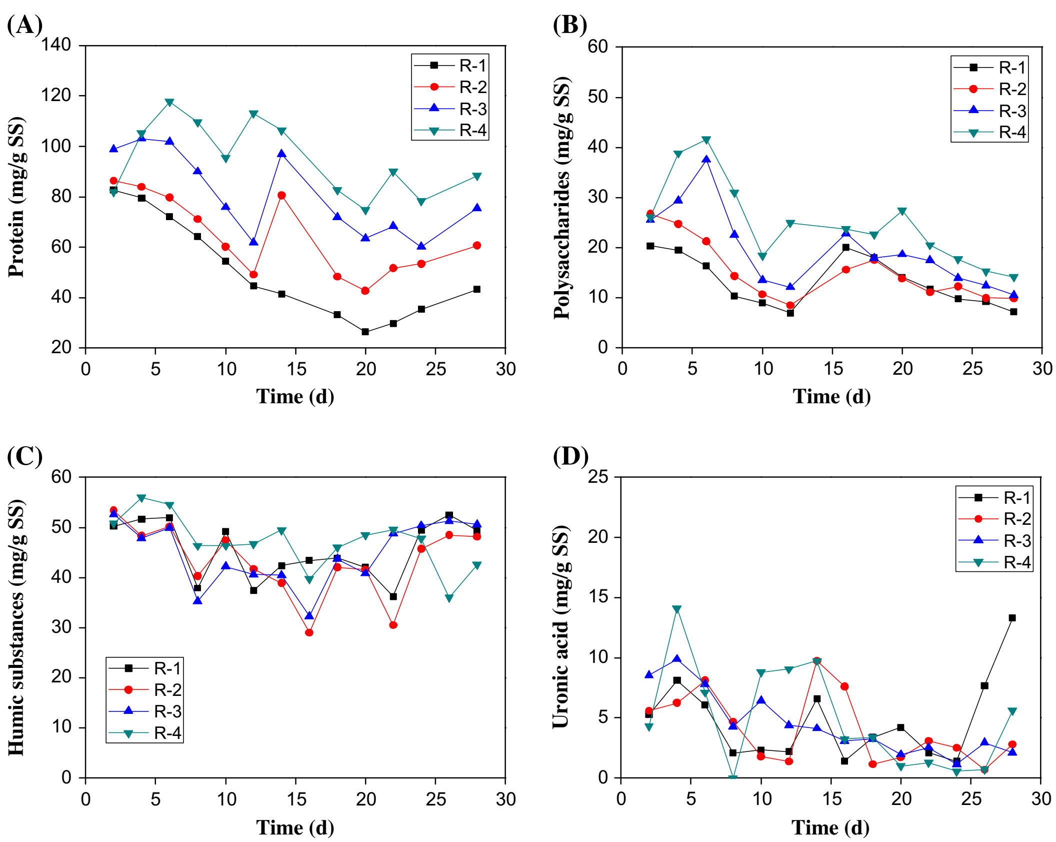


Fig. 5. Variation of sludge EPS components (A) PN, (B) PS, (C) humic substances, and (D) uronic acids in R-1, R-2, R-3, and R-4 during granulation process.

respectively. The results of our studies indicated that higher F/M ratio in R-3 and R-4 produced more EPS as compared to R-1 and R-2 at low influent COD rate on every stage of experiment and a positive correlation with organic loading rate and the amount of EPS produced was observed. The main dominating component of EPS was protein while humic substances were second higher in concentration in all the four reactors throughout this study. This was in contradiction with [31] who observed PS as the second major component in the sludge.

PN and PS exhibited profound changes in their contents during the granulation process. Yi et al. [32] studied the microbial diversity in bio-granulation process and observed changes among the bacterial species during the operation and noted that the synthesis and composition of EPS were dependent on these microbial changes. Protein was the main component of EPS in our studies and an increasing trend in its contents noted during the first week of experiment. The changes in protein contents in SBR system are shown in Fig. 5(a). On day 6, R-4 showed highest protein contents 117.70 mg/g SS which were 1.70 times more than the seed sludge. On the other hand R-2 and R-3 on the same day had 79.75 and 101.95 mg/g SS, respectively which were 1.14 and 1.46 times higher to that of inoculated sludge while R-1 did not show any significant increase in the protein contents and recorded 72.12 mg/g SS (protein was 70.12 mg/g SS in seed sludge of SBRs).The high protein contents in R-3 and R-4 enhanced the granulation process in these reactors as evidenced by the increase in their particle size. Many researchers believe that PN are responsible for the internal stability of aerobic granules. The positive charge of amino groups in PN decreases the overall negative surface charge of EPS. Because of this, the interaction between EPS and microbes increases which help to start the granules formation. The protein contents gradually declined in all the four reactors after the initial rise, and on day 20, these appeared as 26.43, 42.75, 63.56, and 74.81 mg/g SS in R-1, R-2, R-3, and R-4, respectively. These protein concentrations in all reactors were well below than the seed sludge except for R-4 which retained itself around the initial sludge value. The mature granules again showed the growth in protein after this declining period and at the end of studies R-1, R-2, R-3, and R-4 had 43.31, 60.75, 75.50, and 88.31 mg/g SS, respectively.

The changes in PS during granulation process are shown in Fig. 5(b). The trend of variation in PS was almost similar to protein during the first 20 days of studies, but unlike protein the concentrations of PS did not recover in the last phase of experiment and continued decreasing behavior was noted till end. From day 1 to 6, the PS in R-3 and R-4 (operated at 0.32 and 0.43 kg COD/kg MLSS·d) gradually increased to 37.52 and 41.60 mg/g SS, respectively which were 2.16 and 2.39 times of the inoculated sludge (17.40 mg/g SS). The PS in R-3 and R-4 showed a mixed increasing and decreasing trend in their contents from day 6 to 20 and at the end of experiment these were 9.82 and 10.53 mg/g SS, respectively. The PS in R-1 and R-2 (operated at 0.11 and 0.21 kg COD/kg MLSS·d) only increased in the first two days of granulation process and found 20.34 and 26.72 mg/g SS, respectively which were 1.17 and 1.54 times of the seed sludge. After this, these continuously decreased and on day 12, the lowest values 6.95 and 8.49 mg/g SS in R-1 and R-2, respectively were found. Micro-organisms produce more EPS under harsh conditions [33]. The continuous low supply of food in terms of influent COD in these reactors, created stressful conditions which led the microbes to yield more EPS and these increased to 17.99 and 17.56 mg/g SS in R-1 and R-2, respectively on day 18. At the end of studies, the mature granules in R-1 and R-2 had polysaccharide contents 7.16 and 9.82 mg/g SS, respectively.

Protein and polysaccharide concentrations were much lower in the mature granules as compared to the seed sludge but the effect was less in R-4 due to high COD loading rate. The PS in R-1, R-2, R-3, and R-4 were 58.85, 43.56, 39.48, and 18.91%, respectively less in concentration on day 30 to that of inoculated sludge. On the other hand protein contents at the end of experiment were 38.23 and 13.36% less in R-1 and R-2, respectively while R-3 and R-4 were 7.67 and 25.94%, respectively higher than the seed sludge. It indicated that micro-organisms had consumed EPS for food as the source of carbon and energy for their metabolic activities. This effect was more pronounced in R-1 and R-2 as compared to R-3 and R-4 as the formers were already shortage in food due to low F/M ratio than the latters. It is important to note that percentage decrease in PS was much higher than the protein in mature granules. McSwain et al. [15] found that the PS are present on the outer surface of granules and protein in the inner core. This was the prime reason for much reduced PS than PN in the final stage of granulation in our studies because it is easier for micro-organisms to get food from outer layer EPS than the inner layer.

Humic substances showed almost similar contents and trend of variation throughout the granulation process in all reactors. They increased up to 51.94, 50.20, 49.91, and 54.55 mg/g SS in R-1, R-2, R-3, and R-4, respectively from 48.85 mg/g SS of the inoculated sludge on day 6 of experiment. From here onward they exhibited a gradual decreasing trend and observed around 40 mg/g SS in SBRs at the end of day 14. It was very interesting to note that when the granules started to mature, PS and PN were decreasing in their contents as stated earlier but the humic substances started to increase and stabilized around 50.00 mg/g SS till the end of studies as shown in Fig. 5(c). These changes in humic substances showed that different organic loading rates had no effect on their creation and it was presumed that they had no major role in determining the physicochemical properties of granular sludge as their concentration was same in all the reactors irrespective of different F/M ratios. As shown in Fig. 5(d) uronic acid contents like humic substances were also similar in all reactors and varied very little to that of the seed sludge (5 mg/g SS) during the operation and normally found between 5 and 10 mg/g SS. On day four they showed the highest contents and measured 8.12, 6.25, 9.87, and 14.11 mg/g SS in R-1, R-2, R-3, and R-4, respectively and decreased a little afterwards. Uronic acids showed no effect and correlation with different loading rates which indicated their reduced role in the granulation process.

3.4.2. Variation of effluent EPS in SBR system

The effluent samples of all rectors for the determination of EPS contents were daily analyzed and these were called soluble EPS in this study. As shown in Fig. 6(a), during the first six days of operation, PS appeared in effluents and their contents varied between 0.80 and 8.80 mg/L in all reactors and no correlation was found. In second week, when granules were being formed in SBRs, no carbohydrates were detected in any of the effluent samples indicating that they were completely utilized in the formation of granules. But as the granules started to mature and gained stability in the last half of experiment, these again appeared and measured around 6 mg/L in all reactors. Soluble PN were found only on the very first day of studies and noted 3.66, 1.33, 4.00, and 5.66 mg/L in R-1, R-2, R-3, and R-4, respectively. After that they were not found in effluents till 28 day of experiment; this revealed the idea that protein was the principal component of aerobic granules along with carbohydrates and no leakage of protein from sludge was observed. The effluent samples at the end of experiment had 2.75, 0.63, 1.50, and 5.25 mg/L protein

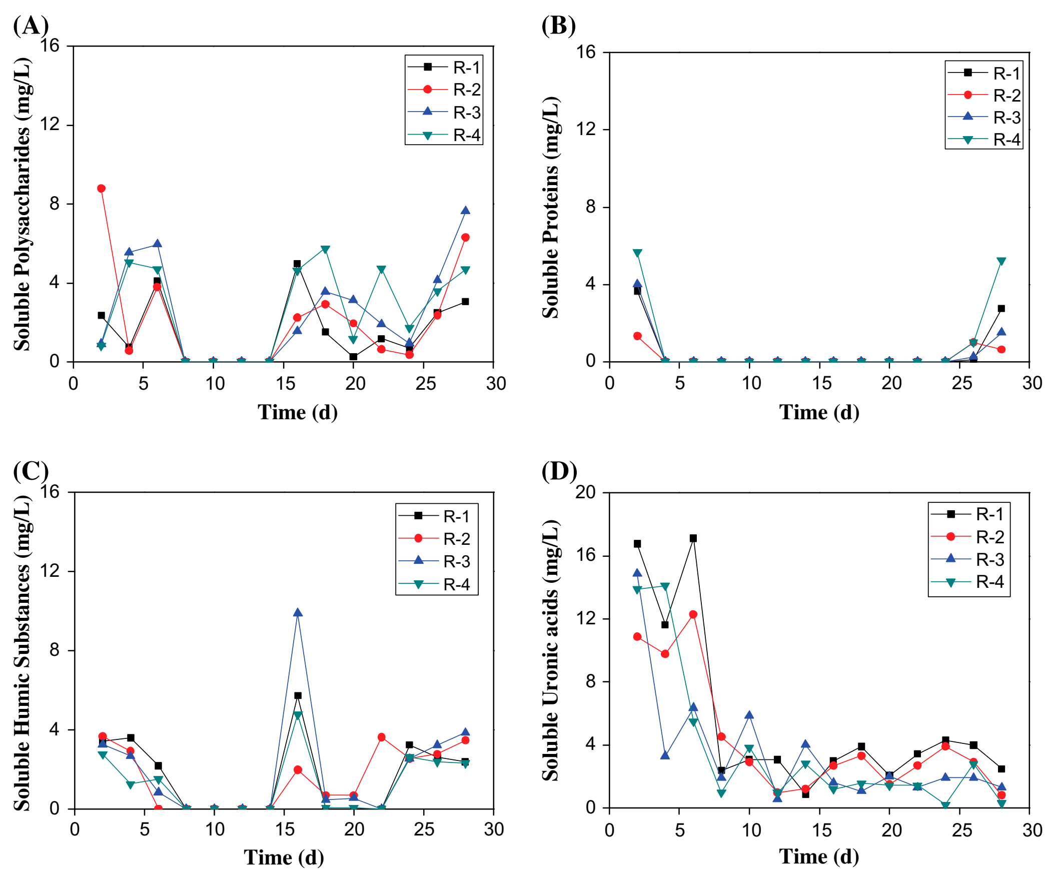


Fig. 6. Changes in contents of soluble EPS (A) PS, (B) PN, (C) humic substances, and (D) uronic acids in effluents of R-1, R-2, R-3, and R-4.

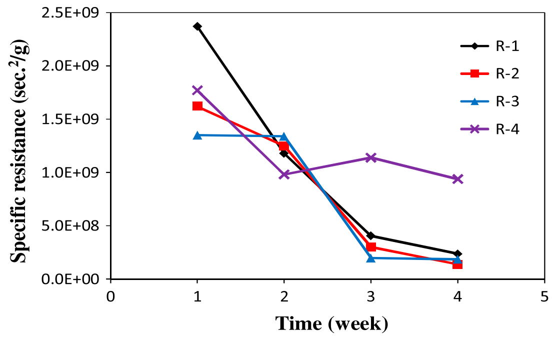


Fig. 7. Comparison of specific resistance values of R-1, R-2, R-3, and R-4 with different F/M ratio.

in R-1, R-2, R-3, and R-4, respectively (Fig. 6(b)). As shown in Fig. 6(c), the humic substances displayed trend similar to PS in effluent samples and appeared in the beginning and then in the last two weeks of operation, their contents varied from 0.51 to 5.72 mg/L in all the reactors.

Fig. 6(d) shows that the behavior of soluble uranic acid was very different than the other three components of EPS in our studies. These were found in the effluent samples of all reactors throughout the entire period of experiment. It was very surprising to note that uronic acid contents were higher in effluent samples as compared to respective sludge samples in the first six days of running. They showed highest contents on day second and measured as 16.77, 10.86, 14.89, and 13.89 mg/L in R-1, R-2, R-3, and R-4 effluents, respectively while the sludge samples in these reactors at the same time were found around 5 mg/L. From day 8, a decrease in their concentration started but still their values were comparable with granular sludge samples and this trend was observed in the whole experiment. Their high contents in effluents suggested that these are not very essential part of aerobic granules and preferred to remain in the soluble form during the operation.

3.5. Specific resistance of granular sludges and its relationship with EPS

Fig. 7 describes the comparison of specific resistance values of reactors during the experiment. The specific resistance to filtration of sludges was measured on regular basis using the Buchner Funnel apparatus during granulation process in order to investigate their filterability and dewaterability characteristics. The specific resistance of seed sludge was $6.80 \times 10^9\ s^2/g$ and a gradual decreasing trend in its value was noted in all the reactors with the progress of experiment. Lower the values of specific resistance, easier is the filtration of sludges and better is their water releasing efficiencies. After one week, the sludge in R-1, R-2, R-3, and R-4 showed the specific resistance values 2.37×10^9, 1.62×10^9, 1.35×10^9, and 1.77v$10^9\ s^2/g$, respectively. These lower values of specific resistance indicated that the dewaterabilities of sludges were improved much efficiently in SBR system as compared to seed sludge, but there was a significant difference among the results of individual reactors. The specific resistance values of R-2 and R-3 were lower than R-1 and R-4 at this stage as stated above. This variation was due to different EPS contents of these reactors which were measured 138, 160, 195, and 215 mg/g SS in R-1, R-2, R-3, and R-4, respectively on the same day. Because of low EPS concentrations in R-1, there was less binding of EPS with the cells and sludge had low floc size and hence unable to remove water easily. R-2 and R-3 showed better specific resistance due to higher EPS values, increase in EPS contents decreased the shear sensitivity and degree of dispersion of sludges which ultimately led to increase their dewatering characteristics [2]. In R-4, too high EPS contents particularly PS affected the water removal abilities of the sludge in this reactor. Neyens et al. [17] suggested hydrogen bonding and electrostatic interaction binding mechanisms between water and EPS molecules. Due to enormous EPS contents in R-4, these two interactions became very significant and sludge trapped much water molecules with it and become difficult to be dewatered, therefore higher specific resistance values observed in R-4 compared to R-2 and R-3.

The polysaccharide contents of R-4 were around 42 mg/g SS which were much higher than the other reactors at same stage. As the PS are hydrophilic in nature and moreover these are present on the outer surface of sludge as mentioned previously. They reduced the rate of filtration by changing the surface properties of sludge. It indicated that granular sludge's water removal capabilities were dependent upon the nature and concentration of EPS and their suitable values are important for the process. Zhou et al. [34] used the FT-IR technique to study the membrane fouling and found PS as one of the natural biofoulent.

With the progress of experiment the dewaterabilities of granular sludges in all the four reactors improved continuously but this trend was lower in R-4 and it was noted throughout the experiment. The high specific resistance values of this reactor were attributed to its high organic loading rate compared to other reactors which resulted in the production of more EPS contents particularly PS. As the high EPS contents increase the viscosity of sludge which resulted in the hindrance of filtration process, so higher specific resistance values were observed.

The values of specific resistance at the completion of experiment were 2.37×10^8, 1.38×10^8, 1.86×10^8, and 9.32×10^8 in R-1, R-2, R-3, and R-4, respectively indicating the enhanced filterability of granular sludges in the SBR system. The appearance of granules in the latter stages of experiment was very effective in reducing the specific resistance of sludges. The compacted particles began to formulate which helped the filtration process. The polysaccharide contents of EPS at this stage were 7.16, 9.82, 10.53, and 14.11 mg/g SS in R-1, R-2, R-3, and R-4, respectively, which were about 58.85, 43.56, 39.48, and 18.91% less to the inoculated sludge. The decrease of PS contents contributed to the relative hydrophobicity of granular sludges which favored its dewaterability. R-2 and R-3 showed better specific resistance values and both had nearly 40% less PS in the granular sludge to the seed sludge but too less and high PS in R-1 and R-4, respectively affected the dewatering abilities of sludge, so it can be suggested that a suitable amount of EPS is very important for their water removal characteristics. It can be seen that R-2 and R-3 operated at moderate F/M ratio produced suitable amounts of EPS which was vital for sludge filtration, while R-4 possessed high EPS values due to increased influent COD and it worsened its dewaterability. The humic substances and uronic acids had almost similar contents in all reactors during the studies, showing that they had no major role related to sludge filtration.

3.6. Relationship among specific resistance, settleability, and PN/PS ratio of granular sludges EPS

As discussed in previous sections, the total contents of EPS increased in SBRs as compared to the inoculated seed sludge during the granulation process. Therefore, the ratio of PN/PS also altered which is very significant in describing the surface properties of sludges. PN/PS in R-1, R-2, and R-3 reached around 6.0 on day 12 while that of R-4 was around 5.0 to that of 4.12 of seed sludge and almost similar trend was noted till the end. PN are considered to be made mostly from glycine etc. which are hydrophobic while sugars in PS have hydrophilic character, therefore the surface characters of sludges are mainly dependent on these groups [4]. The high PN/PS ratio in R-1–R-3 enhanced hydrophobicity of sludges which helped to improve their water removal efficiencies by reducing the specific resistance values. As the protein has positively charged amino groups and PS are negatively charged. This was the reason for enhanced filtration rate in these reactors as the positively charged group neutralized the negatively charged PS which reduced the overall surface charge on sludge and increased its dewaterability. While the low PN/PS ratio in R-4 due to high PS increased its specific resistance values as compared to other three reactors and consequently worsened the filtration rate.

On the other hand the increase in sludge settleability was observed with the decrease of PN/PS ratio in R-4 due to increase of polysaccharide contents which are known as bio-glues and hold the particles together to facilitate the flocculation process. Low values of SVI in R-4 can be attributed to reduced PN/PS ratio in this reactor comparing to other reactors which showed high PN/PS ratio. From the present study, it is suggested that the sludge settleability and filterability are oppositely related with PN/PS ratio. Furthermore, it can be deduced that high organic loading in R-4 increased the total EPS contents while higher PN/PS ratio were obtained in R-2 and R-3 operated at lower F/M ratio.

4. Conclusions

In this study, the variation of EPS and other physicochemical characteristics of granular sludges to the change of influent organic loading were investigated in the SBR system. The important conclusions are as follows.

- The behavior of various components of EPS was very different from each other. The production of PN and PS was positively correlated with the organic loading rate. While, humic substances and uronic acid contents did not show any significant change during the granulation process. However, the total amount of EPS produced was increased with the increase of loading rate in the following order R-4 > R-3 > R-2 > R-1.
- Among the four types of EPS studied, only uronic acids were found higher in effluent samples as compared to their contents in the respective sludge samples which showed their reduced role in the aerobic granules formation.
- The sludge settleability improved with increase of EPS concentrations and influent COD loading rate, therefore R-3 and R-4 showed excellent settleability as compared to R-1 and R-2.
- On the other hand, high organic loading rate in R-4 had a negative effect on the dewaterability of granular sludges due to the increase of polysaccharide contents while R-2 and R-3 (operated at moderate F/M ratio) showed better sludge filtration properties due to moderate amounts of PS.

- The high EPS contents increased the specific resistance values while the SVI_{30} values decreased with an increase in EPS concentration in the SBR system.

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