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# Sequencing Batch Reactor and Bacterial Community in Aerobic Granular Sludge for Wastewater Treatment of Noodle-Manufacturing Sector

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**Abstract:** The sequencing batch reactor (SBR) has been increasingly applied in the control of high organic wastewater. In this study, SBR with aerobic granular sludge was used for wastewater treatment in a noodle-manufacturing village in Vietnam. The results showed that after two months of operation, the chemical oxygen demand, total nitrogen and total phosphorous removal efficiency of aerobic granular SBR reached 92%, 83% and 75%, respectively. Bacterial diversity and bacterial community in wastewater treatment were examined using Illumina Miseq sequencing to amplify the V3-V4 regions of the 16S rRNA gene. A high diversity of bacteria was observed in the activated sludge, with more than 400 bacterial genera and 700 species. The predominant genus was *Lactococcus* (21.35%) mainly containing *Lactococcus chungangensis* species. Predicted functional analysis showed a high representation of genes involved in membrane transport (12.217%), amino acid metabolism (10.067%), and carbohydrate metabolism (9.597%). Genes responsible for starch and sucrose metabolism accounted for 0.57% of the total reads and the composition of starch hydrolytic enzymes including α-amylase, starch phosphorylase, glucoamylase, pullulanase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and 1,4-α-glucan branching enzyme. The presence of these enzymes in the SBR system may improve the removal of starch pollutants in wastewater.

**Keywords:** aerobic granular sludge; sequencing batch reactor (SBR); bacterial community; noodle-manufacturing; wastewater treatment; predicted functional analysis

## 1. Introduction

Among the wastewater treatment processes applied in recent days, sequencing batch reactor (SBR) has been considered as one of the most popular technologies. SBR is a type of activated sludge process that is comprised of aerobic or anaerobic digesters or mechanical biological treatment facilities in batches [1]. These systems generally require large surface areas for treatment and biomass separation units which enable the sludge to settle down, separating from the treated water. Therefore, the settling velocity of activated sludge plays an important role in reducing the remaining time in the settlement/decanting tank, and as a result, boosts the treatment process [2]. Conventional

activated sludge systems are limited, due to the generally poor settling properties of the sludge [3]. To improve the ability to settle, new technology has been developed using aerobic granular sludge. Granules creating aerobic granular activated sludge are considered as aggregates of microorganisms, mainly bacteria, and extracellular polymeric substances [4]. Aerobic granular sludge also functions as a biofilm in suspension, which is composed of combining cells [5]. The application of granules for wastewater treatment has many advantages. They settle significantly faster than activated sludge flocs (high settling velocity). In addition, granular sludge provides high and stable rates of metabolism [5].

Activity of microbial community in granular sludge directly affects the effectiveness of the biological treatment of wastewater. Therefore, it is important to define the microbial composition as well as the interaction between species in an active treatment system [6]. Microbial diversity in a wastewater treatment plant is usually high with the appearance of bacteria, protozoa, metazoa and other microlife [7], but bacteria still do the major role in degrading organic pollutants in the treatment process. It is known that culture-dependent methods have limitations for studying natural microbial community composition, because only a small part of bacteria in environmental samples are culturable under laboratory conditions [8]. Molecular techniques such as polymerase chain reaction–denaturing gradient gel electrophoresis) (PCR–DGGE) [9–11], amplified ribosomal DNA restriction analyses (ARDRA) [12], and fluorescence in situ hybridization (FISH) have been widely applied to study the diversity of microorganisms in natural samples [13]. These techniques have significantly overcome the weakness of traditional methods, but only elucidate the major microbial groups in wastewater treatments. Recently, the metagenomic approach has emerged as one of the most effective and popular techniques for investigating microbial diversity and community in activated sludges [14–18].

The aims of this work are to develop a SBR system for wastewater treatment in a noodle-manufacturing village in Vietnam with high starch concentration, and to investigate the diversity of bacterial communities and their predictive functions presented in aerobic granular sludge, using MiSeq sequencing technique. The sludge used in this study is sampled from the SBR system conducted on a laboratory scale.

## 2. Materials and Methods

### 2.1. Wastewater Sample Collection for SBR System

The wastewater from the noodle-manufacturing village of Phudo–Hanoi was collected and used as input material for the treatment process. The wastewater was characterized by parameters such as chemical oxygen demand (COD): 2500–3500 mg/L; biochemical oxygen demand ($BOD_5$): 1700–2000 mg/L; total nitrogen (TN): 50–70 mg/L and total phosphorous (TP): 25–30 mg/L. The treatment process in the SBR system was supplemented with 50 mL of isolated inoculum *Lactobacillus* sp. ($10^8$ CFU/mL) to increase the density of useful microorganisms.

### 2.2. Operating Conditions of the SBR System

The SBR system in this study had a total volume of 9.42 L and input COD ranging from 0.65 to 3 kg/$m^3$·day). The pH of the wastewater was adjusted and remained at approximately 6.5–7.0, and the reactor was thoroughly stirred by air pumping dissolved oxygen (DO), with an $O_2$ concentration of 5 mg/L, into the system. After the stability of granular sludge, the load of the system maintained a COD of around 4.5–7.5 kg/$m^3$·day. The reactor was continuously operated for three hours for each batch. Initially, each round included 2 min for water and 166 min for air supply, then 10 min for settling and water pumping for another 2 min (Figure 1).

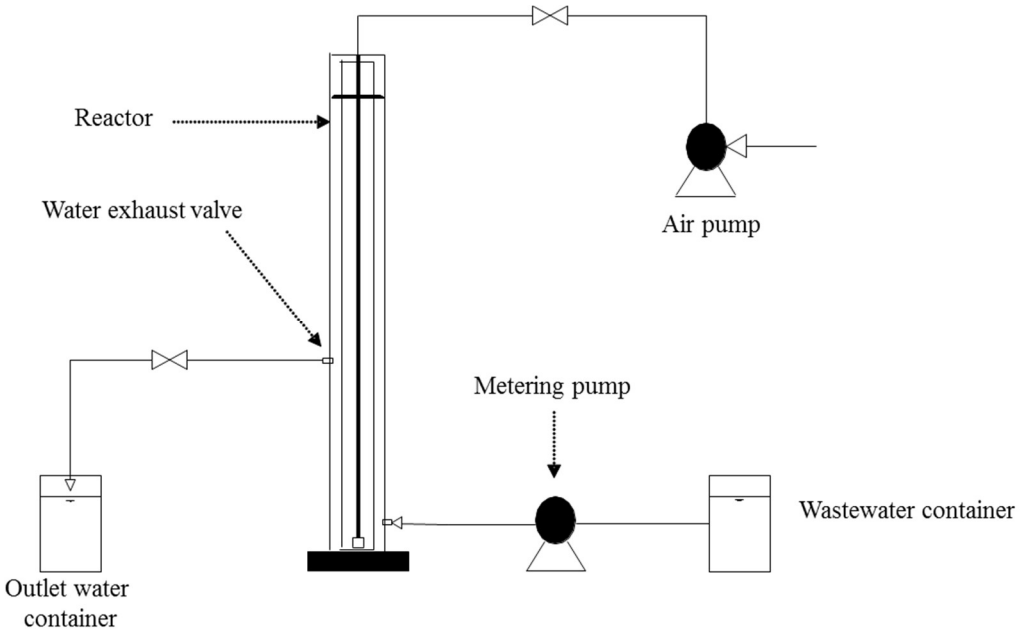


**Figure 1.** Diagram of the sequencing batch reactor (SBR) system. Operation process starts with pumping wastewater through the metering pump into the reactor. Gas is supplied into the reactor through air pump and the outlet water is discharged via exhaust valve and collected in a container.

### *2.3. Collection of Activated Sludge and Outlet Water Analysis*

The outlet water from the SBR system was collected and pooled in a container after one day of operation (equivalent to 8 batches). Then, the outlet water samples were collected daily for analysing parameters such as COD, TN, and TP concentrations according to Standard Methods (APHA-AWWA-WPCF 1998) [19]. The sludge samples were collected from the SBR system and filtered with a membrane of pore size 45 µm (MF Millipore® Membrane, MCE, 0.45 µm, 47 mm). The collected sludge was also used to determine the sludge volume index at 30 min ($SVI_{30}$), according the method of Jenkins et al. [20]. Data were the average values of three repetitions for each analysis.

Scanning electron microscopy (SEM) was used to track the evolution of microbial populations, and the images were taken by FE-SEM, S4800, Hitachi, Japan. Briefly, the procedure is as follows: rinse sludge sample twice with cacodylate 0.1 M and centrifuge at 3000 rpm in 5 min; stabilize sample with 3% glutaraldehyte/cacodylate 0.1 M (pH: 7.2–7.4) in one hour; wash again with cacodylate 0.1 M and treat with $OsO_4$ 1% in one hour; mix sample with ethanol absolute then pour the mixture on aluminum foil, let it dry completely; cover sample with a conductive layer (Pt-Pd) and observe it by FE-SEM, S4800.

### *2.4. DNA Extraction*

Genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (MO Bio, Carlsbad, CA, USA) following the manufacturer's instructions. DNA was purified using the Powerclean® DNA Clean-Up Kit (MO Bio, Carlsbad, CA, USA), and checked for quantity and quality.

### *2.5. Sequencing of 16S Amplicons*

The extracted DNA was amplified using primers 341F and 805R targeting the V3-V4 regions of the 16S rRNA gene, as described by Jeon et al. [21], with modifications as described by Lee and Eom [22]. Sequencing was carried out using an Illumina Miseq sequencing system (Chunlab, Inc., Seoul, Korea).

### 2.6. Bioinformatics Analysis

For metagenomics profiling, reads containing ambiguous bases or low quality bases were filtered. After paired-end reads overlapped, primers were trimmed using pairwise alignment and the Hidden Markov Model. Taxonomic assignment was carried out by comparing the sequence reads against the EzTaxon-e database, using a combination of the initial BLAST-based searches and additional pairwise similarity comparisons. The following criteria were applied for the taxonomic assignment of each read (x = distance values): species ($x \leq 0.03$), genus ($0.03 < x \leq 0.05$), family ($0.05 < x \leq 0.1$), order ($0.1 < x \leq 0.15$), class ($0.15 < x \leq 0.2$), and phylum ($0.2 < x \leq 0.25$). If the distance was greater than the cutoff value, then the read was assigned to an unclassified group. If the sequence cluster could not be identified with a valid name, the accession number of the GenBank sequence entry sharing the highest sequence similarity with the sequence cluster was used as a provisional name. Then the UCHIME algorithm was used to detect chimeric sequences [23]. The obtained high-quality reads were subjected to analyses of the diversity index in the software CLcommunity (Chunlab, Inc., Seoul, Korea).

### 2.7. Functional Predictions

First, a collection of closed-reference operational taxonomic units (OTUs) were obtained from the filtered reads using QIIME v 1.8.0 [24]. The biom-formatted OTUs table was loaded to PICRUSt on the online Galaxy version in the Langille Lab (v1.1.1), alongside the 18 May 2012 Greengenes database, in order to predict genes from the metagenome [25].

## 3. Results

### 3.1. Organic Matter Removal of the SBR System

COD input in the first weeks remained at around 620 mg/L, and treatment efficiency of the system was sustained at 45%. After one month of operation, the efficiency significantly increased and reached around 82%, with an organic loading rate (OLR) of 3.8 kg COD/$m^3$·day, despite the corresponding high amount of COD input (1400 mg/L). After two months, COD removal efficiency increased to 93%, and the OLR value nearly doubled (7.4 kg COD/$m^3$·day) in comparison to the first-month of operation (Figure 2a).

### 3.2. Total Nitrogen (TN) and Phosphorous (TP) Removal of the SBR System

Similar to the COD treatment, the removal efficiency of TN and TP was not high in the first week (22% for N and 28% for P), but after two months of operation, this parameter was gradually increased to around 83% and 75%, respectively. The value of TN loading rate was about five times higher than those of TP, and maintained at this value in the second month. The loading rate value changed correspondingly with N or P removal efficiency and reached the highest amounts of 0.26 kg N/$m^3$·day (Figure 2b) and 0.05 kg P/$m^3$·day (Figure 2c).

### 3.3. Sludge Volume Index ($SVI_{30}$)

In the first two weeks, the $SVI_{30}$ index showed a high value around 250 mL/g with a low settleability of the sludge. This value remained in between 100 mL/g and 200 mL/g in the next two weeks, then gradually decreased and remained at approximately 90 mL/g in the second month of operation (Figure 3) (see "Supplementary Materials" for MLSS data).

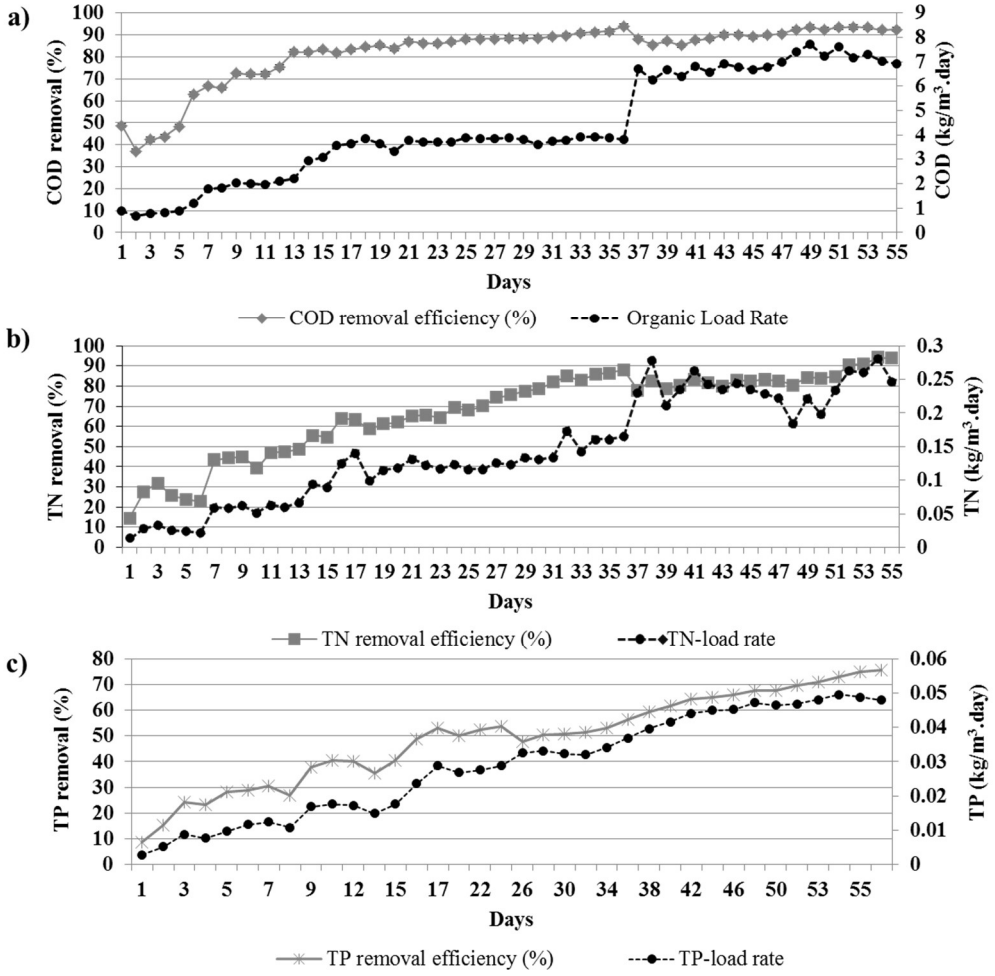


**Figure 2.** Removal efficiency of chemical oxygen demand (COD) (**a**), total nitrogen (TN) (**b**) and total phosphorus (TP) (**c**) in the SBR system.

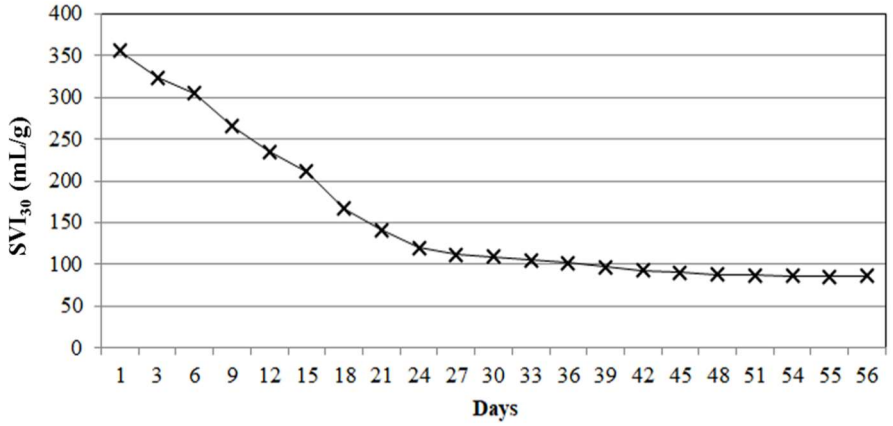


**Figure 3.** $SVI_{30}$ profile of the granules during the two-month operation of the SBR system.

### *3.4. Granular Sludge Structure*

The one-week old sludge sample showed a low density of bacteria and the shapes were varied (Figure 4a). After two months, however, the bacterial density was remarkably increased and the occurrence of ovoid-shaped cells was the most frequent observation in the granular sludge sample, indicating the dominant bacterial strain in the SBR system (Figure 4b).

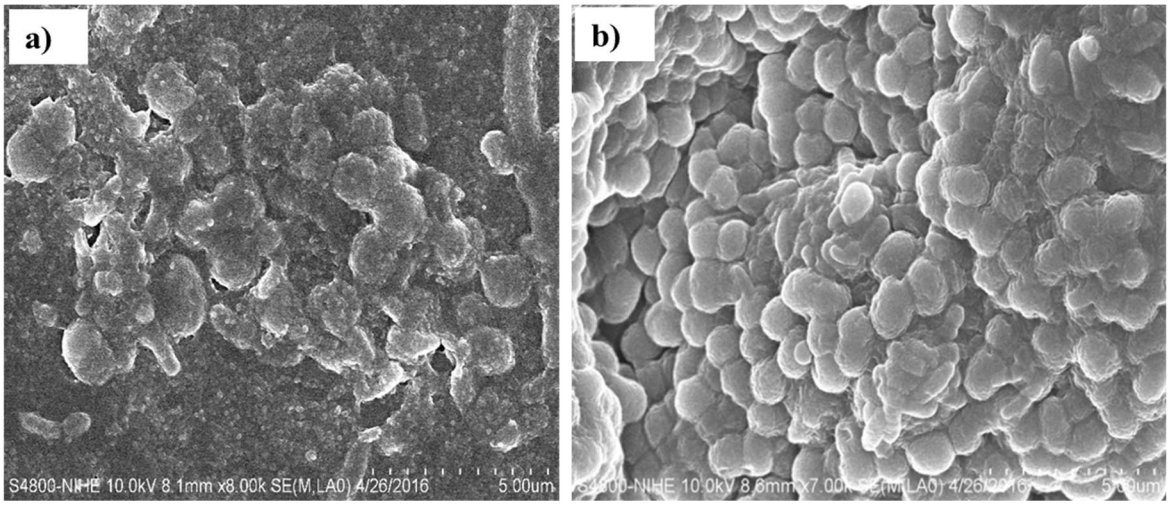


**Figure 4.** Electron microscopy images of granular sludge samples (×8000). SEM images of sludge samples showing the bacterial community were taken at one-week operation (**a**) and eight-week operation (**b**).

### *3.5. Sequencing Analysis*

A total of 42,058 raw sequences were obtained. After removing low quality reads, pair-end merging, trimming of primer and length, de-noising, and discarding chimera reads, 15,590 valid reads remained for further analysis (Table 1). The lowest length of read was 315 bp, the largest one was 461 bp, and the average length was 421 bp.

**Table 1.** Illumina Miseq sequencing and assembly metrics.

| Metrics | Number |
|---|---|
| Total reads | 42,058 |
| Quality trimmed reads | 39,208 |
| Merged reads | 38,545 |
| Primer trimmed reads | 38,355 |
| Length trimmed reads | 38,344 |
| Sampled reads | 20,000 |
| Chimera reads | 4403 |
| Non-target reads | 6 |
| Valid reads | 15,590 |
| Min length | 315 |
| Max length | 461 |
| Average length | 421 |

### *3.6. The α-Diversity*

The sequences were classified into OTUs by setting a 3% distance limit. The rarefaction curve (Figure 5) indicated the number of detected OTUs increased with the number of sequences sampled in the aerobic granular sludge sample. The curve nearly reached an asymptote, which showed that most of the species present have been detected.

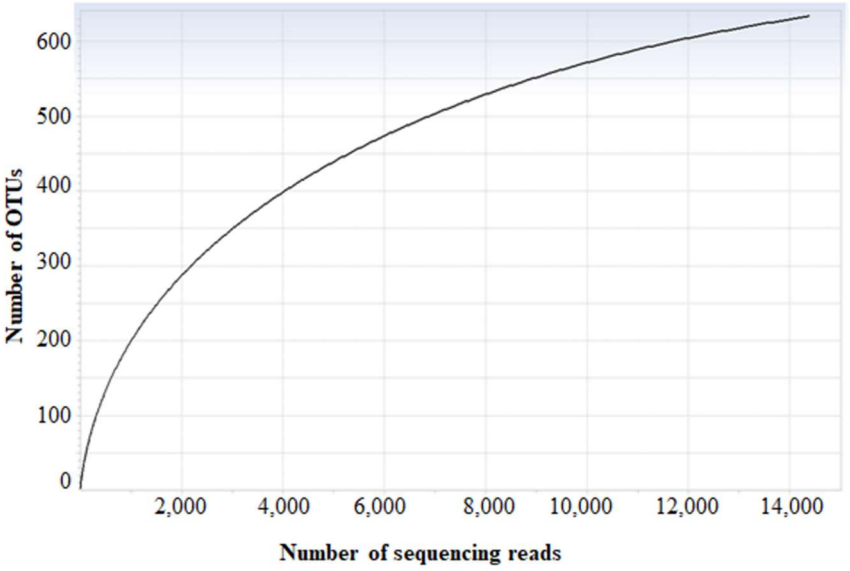


**Figure 5.** Rarefaction curve. OTUs are shown at the 3% genetic distance levels.

The observed OTUs, richness estimators (Ace and Chao1) and diversity estimator (Shannon) of the sample are summarized in Table 2. Ace and Chao1 measures expected OTUs in the sample. Shannon estimates species richness and evenness. The high value of the Shannon index indicates that the high number of species and the distribution of individuals among the species become even. The number of estimated OTUs (Ace and Chao1) in the sample was similar to the number of observed OTUs.

**Table 2.** Diversity indices obtained at a genetic distance of 3%.

| Target Reads | Value |
|---|---|
| Valid reads | 15,590 |
| OTUs | 633 |
| Ace | 751 |
| Chao1 | 701 |
| Shannon | 4.0 |
| Goods Lib. Coverage | 99.0 |

### *3.7. Overall Bacterial Community*

Mi-sequencing data revealed great bacterial diversity in the sample examined. The results showed the bacterial community belonging to 22 phyla, 43 classes, 88 orders, 196 families, 432 genera, and 768 species.

The phyla *Proteobacteria* (relative abundance 34.71%), *Firmicutes* (31.01%), and *Bacteroidetes* (30.71%) were detected as the most diverse (Figure 6a). At the family level, 74.71% of the sequences were assigned to known taxa of *Streptococcaceae* (21.57%), *Prevotellaceae* (9.33%), *Oxalobacteraceae* (6.95%), *Veillonellaceae* (6.18%), *Acetobacteroides* (5.23%), *Pseudomonadaceae* (5.12%), *Campylobacteraceae* (4.41%), *Flavobacteriaceae* (3.81%), *Comamonadaceae* (3.14%), *Porphyromonadaceae* (2.30%), *Bdellovibrionaceae* (2.09%), *Prolixibacteraceae* (1.58%), *Dyadobacter* (1.50%), and *Xanthomonadaceae* (1.50%) (Figure 6b). At the genus level, there were 14 genera which accounted for 67.05% and the most abundant genus was *Lactococcus* (21.35%). At the species level, *Lactococcus chungangensis*, *Janthinobacterium lividum*, and *Megasphaera cerevisiae* were dominant in the sample (Table 3).

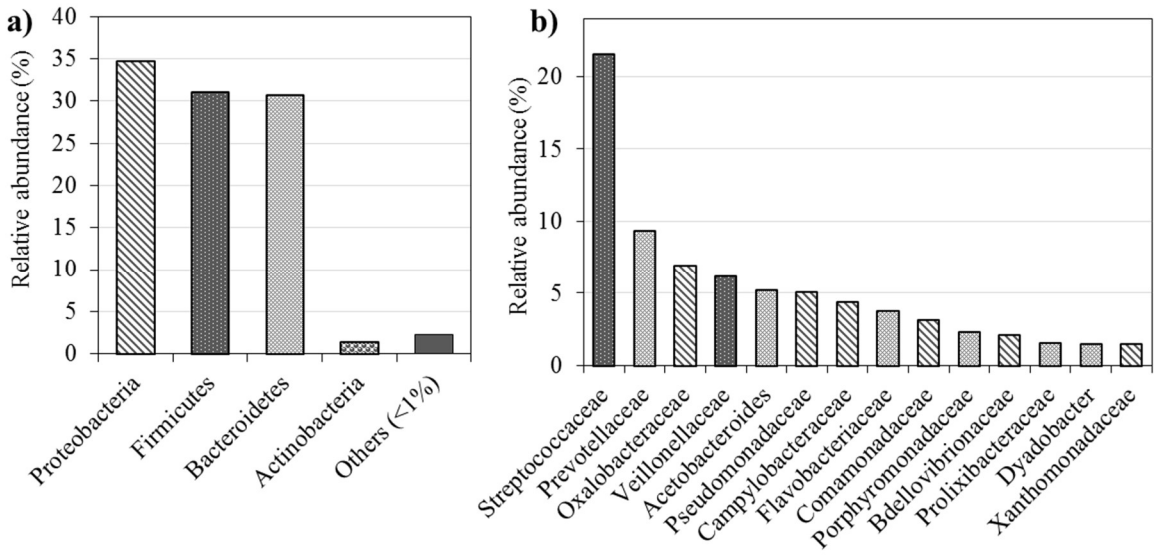


**Figure 6.** Bacterial composition in the granular sludge sample at phylum level (**a**) and family level (**b**). The families that accounted for greater than 1.5% of reads were shown in the figure.

**Table 3.** List of the most frequently occurring species in the granular sludge sample.

| No. | Species | Frequency | Quantity |
|---|---|---|---|
| 1 | *Lactococcus chungangensis* | 20.69120 | 3227 |
| 2 | *Janthinobacterium lividum* | 6.50167 | 1014 |
| 3 | *Megasphaera cerevisiae* | 3.63555 | 567 |
| 4 | *Sulfurospirillum arsenophilum* | 3.59066 | 560 |
| 5 | *Pseudomonas flavescens* | 2.34034 | 365 |
| 6 | *Pseudomonas veronii* | 2.03898 | 318 |
| 7 | *Macellibacteroides fermentans* | 1.76327 | 275 |
| 8 | *Soonwooa buanensis* | 1.10926 | 173 |

### *3.8. Functional Predictions using PICRUSt*

On the basis of the annotation of functional genes using PICRUSt, 41 gene families were identified in the sample, but only families with an abundance > 1% are shown in Figure 7a. The sample was enriched for functions involved in membrane transport (12.217%), amino acid metabolism (10.067%), and carbohydrate metabolism (9.597%) (Figure 7a).

A deep examination of the carbohydrate metabolism revealed that pyruvate, amino sugar and nucleotide sugar metabolisms, glycosis/gluconeogenesis, and butanoate metabolism were major categories (Figure 7b). Putative genes responsible for starch and sucrose metabolism were moderately accounted for 0.57% of the total reads. In addition, subgroup genes such as galactosidase metabolism (0.472%) and glycosis/gluconeogenesis (0.986%) were potentially involved in the starch degradation.

Biological starch removal is one of the most important functions in the activated sludge. In the present study, several starch hydrolytic enzymes were detected in the SBR system such as starch phosphorylase (0.043%), α-amylase (0.043%), glucoamylase (0.001%), pullulanase (0.017%), α-galactosidase (0.036%), β-galactosidase (0.072%), α-glucosidase (0.060%), β-glucosidase (0.113%), 1,4-α-glucan branching enzyme (0.043%), and oligo-1,6-glucosidase (0.002%) (Table 4). By analysing Kyoto encyclopedia of genes and genomes (KEGG) metabolism maps, we discovered that all enzymes were involved in the glycolysis pathway to create pyruvate from starch (Figure 8). Starch was converted by the actions of α-amylase and glucoamylase to form α-D-glucose; and the actions of 1,4-α-glucan branching enzyme and starch phosphorylase to form α-D-glucose-1-phosphate. The enzyme hexokinase catalyzed the conversion of α-D-glucose to

α-D-glucose-6-phosphate. Other enzymes that were involved in the glycolytic pathway included phosphoglucomutase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, phosphoenolpyruvate carboxylase, and phosphoenolpyruvate carboxylase. Pyruvate was converted into acetyl-CoA in the presence of pyruvate dehydrogenase in order to enter the citric acid cycle. Additionally, L-lactate dehydrogenase catalyzed the reversible conversion of pyruvate to L-lactate.

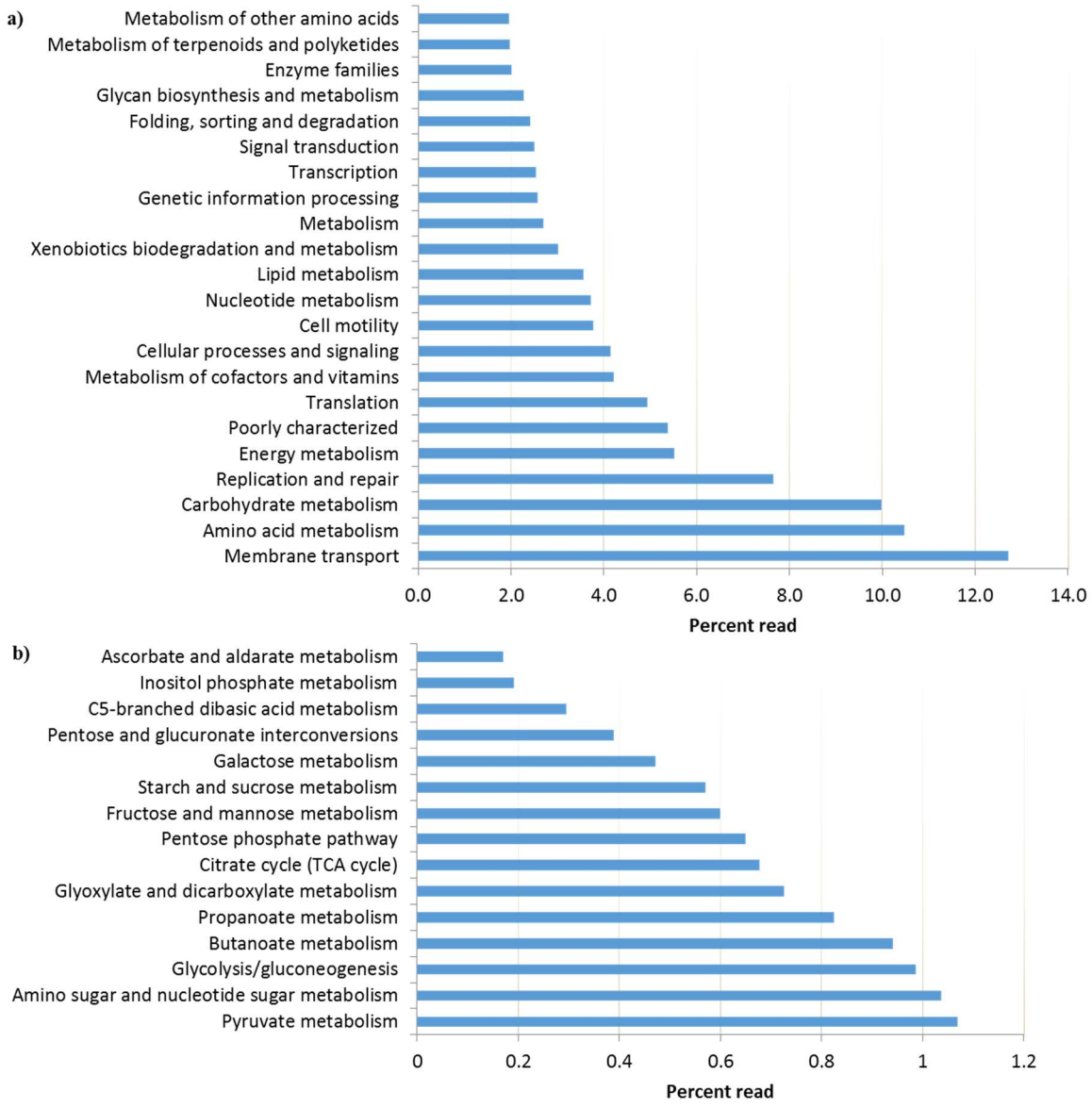


**Figure 7.** Functional analysis based on the Kyoto encyclopedia of genes and genomes (KEGG) subsystem level II (**a**) and carbohydrate metabolism (**b**). At KEGG subsystem level II, only functions with percent read > 1% are shown.


**Figure 8.** Pathway from starch to pyruvate in the SBR system. Starch was metabolized to pyruvate through glycolysis. All enzymes were present in the SBR system.

**Table 4.** Starch hydrolytic enzymes detected in the granular sludge sample.

| Enzymes | Percent Read | Pathway |
|---|---|---|
| Starch phosphorylase | 0.043 | EC:2.4.1.1 |
| α-amylase | 0.043 | EC:3.2.1.1 |
| Glucoamylase | 0.001 | EC:3.2.1.3 |
| Pullulanase | 0.017 | EC:3.2.1.41 |
| α-galactosidase | 0.036 | EC:3.2.1.22 |
| β-galactosidase | 0.072 | EC:3.2.1.23 |
| α-glucosidase | 0.060 | EC:3.2.1.20 |
| β-glucosidase | 0.113 | EC:3.2.1.21 |
| 1,4-α-glucan branching enzyme | 0.043 | EC:2.4.1.18 |
| Oligo-1,6-glucosidase | 0.002 | EC:3.2.1.10 |

## 4. Discussion

The first two weeks were the acclimatization process of bacteria, and then the formation of granules began. This explained the low treatment efficiency of both COD and TN removal in the initial

process. These parameters significantly increased in the following weeks after the lag phase, despite the fact that the load of COD input also increased. According to the investigation of Dockhorn et al. [26], which used a SBR system to treat municipal wastewater, the efficiency of COD removal was also remarkably high after the acclimatization process of bacteria in the system, which grew to 94.5% after 20 days. Similar results were also reported in previous studies [27–29]. The value of the organic load rate (OLD) in this study was significantly higher, reaching 7.4 kg COD/$m^3$·day when the system was in a stable state, which was two times higher than the similar operating SBR system for municipal wastewater treatment reported by Jungles et al. [30]. This could explain why the wastewater from the noodle-manufacturing village contained more easily degradable organic matter such as starch, in comparison to municipal wastewater. However, the loading rate for N was higher in the case of municipal wastewater, as compared to the noodle-manufacturing wastewater at 0.36 g N/L·day and 0.26 g N/L·day, respectively.

In an aerobic granular wastewater treatment system, the removal efficiency of TN is normally higher than that of TP through the nitrification process carried out by living microorganisms, which could eventually produce $N_2$ gas from the treatment system. Most of the phosphorous is taken up and transformed into microorganism biomass and/or precipitated by complex components within active sludge, and remains in the treatment system [31,32]. The gasification of TN (mostly in the form of ammonia) to $N_2$ gas is conducted by microorganism due to anoxic condition created by granular activated sludge, which a conventional aerobic wastewater treatment is usually limited [33]. The dominance of TN over TP removal efficiency was also observed in this study, but the difference was not significant (83% vs. 75%). The TN and TP removal rate reached a high value indicating that the settleability of the sludge in the SBR system was relatively fast and effective, and led to a reduction in the suspended microorganism biomass, which mainly contributed to a high nitrogen and phosphorous concentration in effluent [34,35]. This was reflected by $SVI_{30}$ values, a parameter describing the ability of the sludge to settle and compact, that gained the optimal range from 100 mL/g to 200 mL/g after two weeks and was sustained around 90 mL/g for the rest of the operational periods. At this range, the sludge was characterized by a quick settling and high compact, and more importantly, pulling down more particulate matters while settling to produce a clear and good quality of treated water. The $SVI_{30}$ value usually performs a higher value (more than 250 mL/g) when a system is undergoing startup and the sludge is just formed, meaning that the sludge has a poor and slow settleability. This happened in the first two-week operation of the SBR system in this study. In some cases, a filamentous sludge bulking could also make the $SVI_{30}$ value high, because of filaments trapped within the floc, causing a dispersed and open structure leading to the decrease the settleability of the sludge [20].

A total of 633 OTUs was obtained from 15,590 valid reads indicating that the SBR system was a low species-rich environment, as compared to other wastewater treatment plants [36–38] but was similar to the other SBR system [39]. The abundances of *Proteobacteria* and *Bacteroidetes* in the sample were consistent with previous studies [38,40,41]. The high relative abundance of *Firmicutes* was typical for this sample. The species *L. chungangensis* (belonging to the family *Streptococcaceae*) with the highest occurrence was characterized by Gram-positive, coccoid or ovoid-shaped, non-spore-forming and non-mycelium-forming, which was similar to the shape of bacteria in granular sludge captured by electron microscope in this study (Figure 6). This species could grow under aerobic condition or facultative anaerobic condition [42]. The species was also isolated from activated sludge foam from the wastewater treatment plant in Cheonan, Republic of Korea [43]. It has been demonstrated that members of the genus *Lactococcus* utilized starch and produced L-lactate [44–46]. Therefore, the results suggested that *L. chungangensis* contributed maily in starch degradation in the SBR system.

As wastewater containing high level of starch, the elimination of starch pollutants was focused on this study. The biodegradation of starch requires multiples of enzymes. Enzymes converting starch presented in the SBR system, including α-amylase (EC:3.2.1.1) which hydrolysed α-1,4 linkages and formed dextrins; 1,4-alpha-glucan branching enzyme [EC:2.4.1.18] which formed α-1,6-glucosidic bonds; glucoamylases (EC:3.2.1.3) and α-glucosidase (EC:3.2.1.20) which cleaved both α-1,4 and α-1,6

linkages and produced only glucose. However, β-amylase (EC:3.2.1.2) which cleaved α-1,4 and created maltose and β-dextrins was lacking in the SBR system. It was interesting, though, that pullunase [EC:3.2.1.41] which cleaved α-1,6-glucosidic bonds in amylopectin and/or glycogen, was detected in this study. This enzyme was found in many *Bacillus* sp. [47]. The use of pullunase was alongside low glucoamylase enhanced production of D-glucose from starch [48]. Overall, the presence of these enzymes in the SBR system might improve the removal of starch pollutants in wastewater.

## 5. Conclusions

In this study, the SBR system for wastewater treatment of a noodle-manufacturing village in Vietnam with high starch concentration was operated. The COD, TN and TP removal efficiency of the process reached 92%, 83%, and 75%, respectively, after two months of operation. The MiSeq sequencing results showed a significantly high diversity of microorganisms in the granular sludge with more than 400 bacterial genera and 700 species. The most dominant *Lactococcus* species formed granular formation in the SBR system. Functional prediction analyses showed that multiple genes involved in starch degradation were detected in the SBR system. This work has provided information for a more comprehensive understanding of the wastewater treatment method based on microbiological approach.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2076-3417/8/4/509/s1.

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**Author Contributions:** T.T.C. and L.T.T.H. conceived, and designed the study. P.D.H., N.Q.H., and N.N.L. performed the experiments. T.T.C., L.T.T.H., P.D.H., N.S.N. and N.N.L. analysed the data. B.V.C., and L.T.T.H. wrote the manuscript with input from all authors.

**Conflicts of Interest:** The authors declare no conflicts of interests.

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