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Biological Denitrification in High Salinity Wastewater Using Semen Litchi as a Carbon Source

Hua Li[1,2], Ziming Zhou[1,2*], Qingsong Liu[1], Hongbiao Dong[1], Yafei Duan[1], Chunhou Li[1], Jiasong Zhang[1], Hongxin Tan[2]

(1.Key Lab of South China Sea Fishery Resources Exploitation & Utilization, Ministry of Agriculture; South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Guangzhou, 510300, China.

2.College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, 201306, China.)

Abstract: A new agricultural waste semen litchi was used as the sole carbon source to remove nitrate from high salinity wastewater in laboratory reactors. The main nutrient components, the content of heavy metals and the morphology of semen litchi were first studied. The results showed that semen litchi contained about 60% organic carbon source and low levels of heavy metals. The milled semen litchi had lots of gap structure and abundant starch granules. Then the release velocity of carbon source and the denitrification rate in high salinity wastewater were investigated. It was found that semen litchi could supply continuous organic carbon source for denitrification. And the maximum TOC concentration could reach to 137.29 mg/l at 46th day. The nitrate removal rate and denitrification rate could reach to 98.8-99.5% and 192 mg N/(l d), respectively. During the whole denitrification reaction, the nitrite concentration was lower than 0.01 mg/l. Microbial community profile by Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) indicated that the denitrifying bacteria (*Sphingomonas* family and *Rhodospirillum* family) became enriched in the semen litchi sludge. Furthermore, salinity didn't have negative effect

[1] Corresponding author, E-mail: jiasongzhang@hotmail.com

* These authors contributed to the work eqully

on denitrification. Semen litchi could be used as an economical and effective carbon source for denitrification in high salinity wastewater.

Keywords: semen litchi; carbon source; denitrification; high salinity wastewater

1. Introduction

Increased nitrogen pollutants have caused serious eutrophication and algal blooms in most areas of china[1]. Physical, chemical and biological methods have been used to remove nitrate from wastewater. However, among these various methods, heterotrophic denitrification seems to be an environment-friendly and economic process[2].

In the biological denitrification processes, organic carbon source is need as the electron donor for the reduction of nitrate and nitrite transformation into nitrogen gas. Therefore, it is great important to add organics into denitrifying systems, especially in the wastewater with lower C/N ratio[3]. External carbon sources have been widely used in laboratory and engineering applications, such as methanol[4], ethanol[5], acetic acid[6] and biodegradable polymers (BDPs), such as Polylactic acid (PLA)[3], poly-β-hydroxybutyrate (PHB)[7] and polycaprolactone(PCL)[8]. However, liquild carbon source has a risk of overdosing which would cause deterioration of effluent quality and the BDPs are too expensive to be used in engineering application[9].

Natural, organic substances such as rice husk[9], wood chips[10], wheat straw[11] and cotton[12] have been developed as cheap and safe carbon source to remove nitrate from wastewater. Litchi is a tropical fruit of high commercial value in the international fruit market, with an established production rate of 2,600,000 ton/a[13,14]. As the agricultural

waste of litchi, the output of semen litchi is also very large. It is reported[15] that the main component of semen litchi is starch which can provide nutrition for microorganism. However, there are only a few applications of semen litchi in traditional chinese medicine. Little information is currently available to the behaviors in wastewater treatment by natural semen litchi, especially in the high salinity wastewater.

In this paper, we propose semen litchi as the sole carbon source for biological denitrification in high salinity wastewater. The main nutrient components, the content of heavy metals and the morphology of semen litchi was studied firstly. And then the release velocity of carbon source, denitrification rate in high salinity wastewater and the microbial community composition of sludge after denitrification reaction was systematically investigated, respectively.

2. Materials and Methods

Pretreatment of Semen litchi: Semen litchi was purchased from a pharmacy in Guangzhou city. The semen litchi was washed with tap water before air drying (85 ^{o}C). Then semen litchi was smashed into granules of 150-200 mesh. The material was preserved at room temperature (25 ^{o}C) and kept in a moisture-free container.

Total organic carbon (TOC) concentration in Semen litchi Lixivium: The carbon release process was determined in 15 ml centrifuge tubes, 10 ml distilled water and a certain amount of semen litchi were packed in each reactors. The centrifuge tubes were placed in a shaking incubator at 150 rpm (30 ^{o}C). After the 1st, 2nd, 3rd, 4th, 8th, 18th, 32th and 46th day, the lixivium of semen litchi was filtered through

0.45 μm membrane, respectively. Then these samples were analyzed by dissolved organic carbon analyzer (Elementar Liqui-TOC, Germany).

Denitrification processes: The denitrification processes were carried out in 1000 ml Erlenmeyer flasks which were placed on a magnetic stirring apparatus with rotation speed of 200 rpm at room temperature. Eight grams of semen litchi and 1000 ml of synthetic salinity wastewater were mixed with denitrifying activated sludge (the final concentration was 1.5 g/l MLSS). The denitrifying activated sludge (feed sludge) was collected from a recirculating aquaculture system for marine fish. The pH of influent was kept at 7.0-7.5 and salinity was kept at 25‰. The DO level in reactor was less than 0.5 mg/l.

The synthetic high salinity wastewater was prepared as follows[16]: sodium chloride (NaCl, 23.93 g/l), potassium chloride (KCl, 0.68 g/l), calcium chloride ($CaCl_2$, 0.99 g/l), magnesium chloride ($MgCl_2$, 6.09 g/l), magnesium sulfate ($MgSO_4$, 3.94 g/l), sodium bicarbonate ($NaHCO_3$, 0.19 g/l), potassium bromide (KBr, 0.10 g/l), sodium nitrate ($NaNO_3$, 0.364 g/l) and monopotassium phosphate (KH_2PO_4, 0.044 g/l) in tap water. The concentrations of NO_3-N and PO_4-P were about 60 mg/l and 10 mg/l, respectively. The wastewater was replaced every day. Samples were taken and filtered through 0.45 μm membrane before analysis. The concentrations of COD, NO_3-N, NO_2-N were measured according to standard methods[17] every day. The pH was determined with a digital, portable pH meter (OHAUS, ST10, USA). The DO level was measured with a digital, portable DO meter (YSI, Model 55, USA). All the reagents used were analytical purity.

Characterization of Semen litchi: The morphology of semen litchi was examined by scanning electron microscopy (SEM) (TM3000, Hitachi Ltd., Japan). Fourier transform infrared (FTIR) spectrum of semen litchi was recorded using a FTIR spectrometer (IRAffinity-1, Shimadzu, Japan.). The content of main nutrient components was determined by standard methods and heavy metals in semen litchi were determined by Atomic Absorption Spectrometry[18,19].

Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis: The samples of seed sludge and the sludge after denitrification were collected. DNA extractions were completed using ZR Soil Microbe DNA MiniPrep™ Kit, following the manufacturer's recommendations. PCR amplification of V3 16S rRNA genes and DGGE as described by reference[20]. The PCR mixture consisted of 25 μl of 2× KOD Fx Buffer, 10 μl of 10 mM dNTP mixture, 1.5 μmol of each primer, 1.0 μl of KOD Fx, 2.0 μl of DNA extracted from the sludge sample and sterile ultrapure water to a final volume of 50 μl. Specifically, the PCR conditions were: a hot start of 2 min at 94 ℃ followed by 25 cycles of 98 ℃ for 10 sec, 58 ℃ for 15 sec and a final extension step of 1 min at 68 ℃. Electrophoresis was then performed in 1 ×TAE buffer for 16 h at a constant voltage of 100 V at 60 ℃. The gels were then stained for 30 min using GeneFinder (diluted by 10000 ×) and photographed.

Nucleotide sequences were then submitted to a BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve the closest known alignment identities for the partial 16S rRNA sequences. Statistical comparison of the DGGE

patterns on the same gel was completed with Quantity One 4.6.2 (Bio-Rad). Calculation of the similarity matrix was based on the Pearson product moment correlation coefficient. The clustering algorithm was used to calculate dendrograms.

3. Results and Discussion

3.1 The constituents of semen litchi

In this paper, the main nutrient components and the content of heavy metals (Cu, Pb, As, Cd and Cr) in semen litchi were studied firstly. As shown in Tab. 1, high content of starch (48.8%) was determined in semen litchi, similar to the other report[15]. The total organic source of semen litchi could reach to 60.07%, the large amounts of strach coulded be used more easily and quickly by denitrification bacteria. Furthermore, all heavy metals in semen litchi were in low levels and the concentration of Cu was 10.6 mg/kg, Pb (0.013 mg/kg), As (0.017 mg/kg), Cd (0.020 mg/kg) and Cr (0.73 mg/kg), respectively. According to the standard method[18,19], the content of heavy metals were safe in semen litchi for animal feeding stuffs. These results indicated that compared with traditional liquid carbon sources, it was safer and possible to use semen litchi as a substrate in wastewater denitrification.

Tab. 1. The main nutrient components of semen litchi

Item	Content(%)	Methods
Starch	48.8	ISO 15914-2004[21]
Crude protein	4.83	ISO 1871-1975[22]
Crude fiber	3.3	ISO 5498-1981[23]
Reducing sugar	2.5	ISO 5377-1981[24]
Crude fat	0.64	ISO 6492-1999[25]

3.2 FTIR spectra of semen litchi

FTIR spectra of semen litchi were presented in Fig. 1. The broad band at 3356 cm^{-1} was due to the stretching mode of the O-H groups. An intense band at 1648 cm^{-1}

was assigned to the first overtone of the O-H bending vibration. Moreover, the bands at 1159 and 2932 cm^{-1} were assigned to C-O stretching and C-H stretching, respectively[26]. Two strong bands at 1089 and 1014 cm^{-1} were attributed to CH_2-O-CH_2 stretching vibrations and the band at 860 cm^{-1} was assigned to C-O-C ring vibration[27]. The FTIR spectra of semen litchi were very similar to the spectra of starch[28] which was contributed to the large amounts of starch in semen litchi (Table 1). Furthermore, semen litchi had a variety of hydrophilic groups as shown in Fig. 1, which made it had better biocompatibility and could be used as an excellent carrier for denitrifying bacteria.

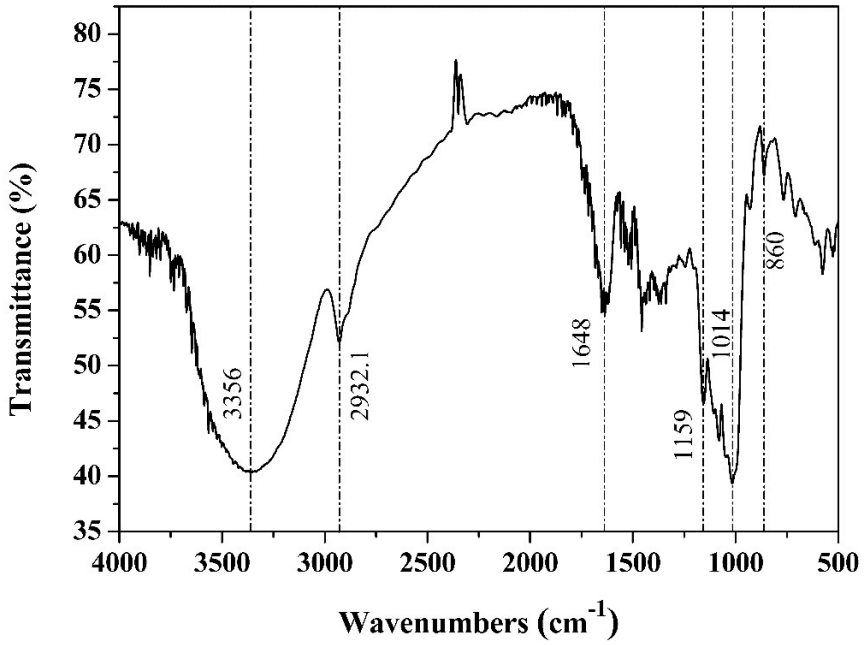

Fig. 1 FTIR spectra of semen litchi

3.3 SEM analysis of semen litchi

The photographs and SEM micrographs of semen litchi were presented in Fig. 2. As can be seen from the Fig 2a, semen litchi had a regular kermesinus oval shape and a smooth surface, which made it harder for bacteria to latch onto. After milling

process, the smooth surface was destroyed and the semen litchi granules become angular-shaped (Figs. 2b). In Fig. 2c, the internal structure of semen litchi was totally exposed and the surface of semen litchi granules become more rough, which made it easier for bacteria to adhere. From the magnified SEM images of semen litchi (Figs. 2d-f), a large number of starch granules with oval and regular shapes could be clearly observed [29,30](Fig. 2f), the gap structure between the starch granules could provide a lot of space for bacteria growth (Fig. 2d). Moreover, the major particle lengths of the starch granules were ranged from 7 to 14 μm (Fig. 2e), and such scale was in the range of cereal starch, but larger than that of rice starch from 2.4 to 5.4 μm[31], and smaller than most other cereal starch, such as corn starch and wheat starch with mean sizes as 10 μm and 18 μm, respectively[30]. The SEM results indicated that after milling process, the rough surface, the abundant starch granules and plenty of gap structure of semen litchi could be benefit to accelerate the adhesion and growth of denitrifying bacteria.

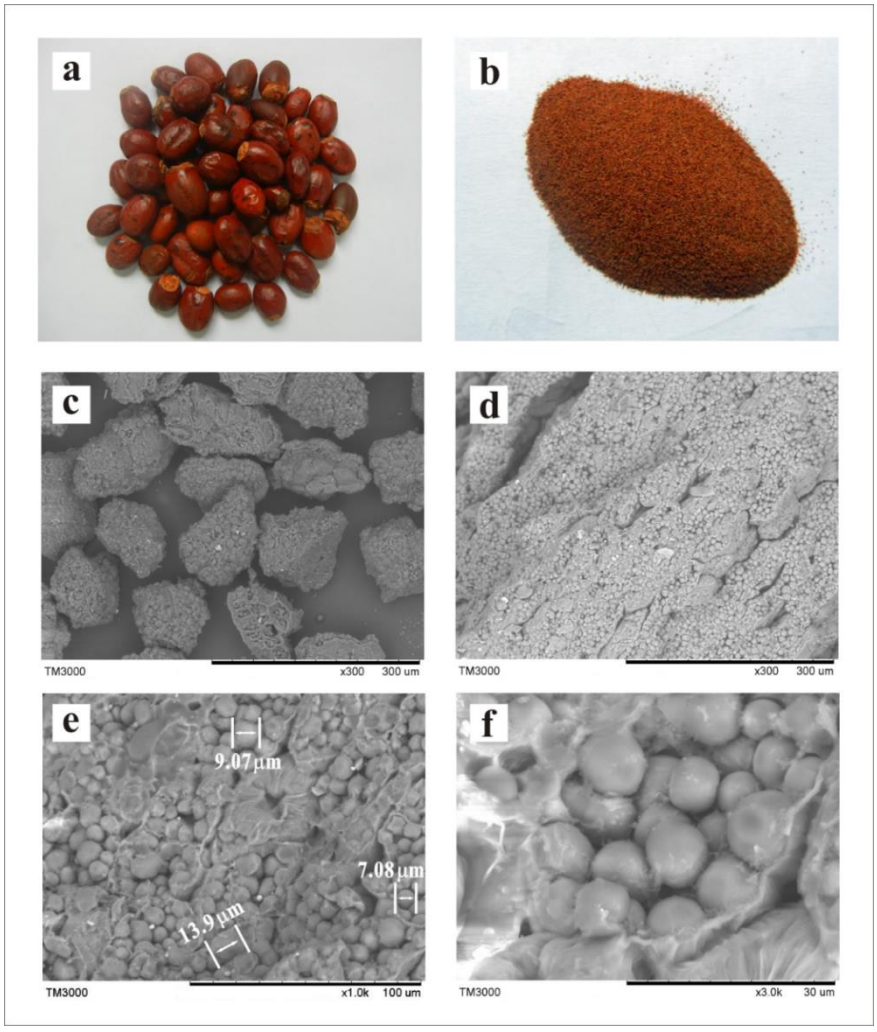

Fig. 2 Photographs (a, b) and SEM images (c-f) of semen litchi

3.4 TOC concentration in lixivium of semen litchi

The TOC concentrations in lixivium of semen litchi at different solid-liquid ratios were shown in Fig. 3. For the first 4 days, TOC concentrations increased quickly, then the release velocity was slow down at 4th-8th days, at last it become stable from the 18th to 46th day. During the initial days of operation, the easily dissolved part of semen litchi (for example reducing sugar) was released out, so the TOC increased quickly. As time went on, the starch and protein were gradually released out resulted in slowly increase of TOC. Finally, the TOC concentration reached to a stable level[32], the TOC concentration of different solid-liquid ratios was

131.23 mg/l, 134.53 mg/l, 135.23 mg/l and 137.29 mg/l, respectively at 46th days. The results of carbon release showed that semen litchi could provide continuous organic carbon source and could be used as an economical and effective carbon source for denitrification.

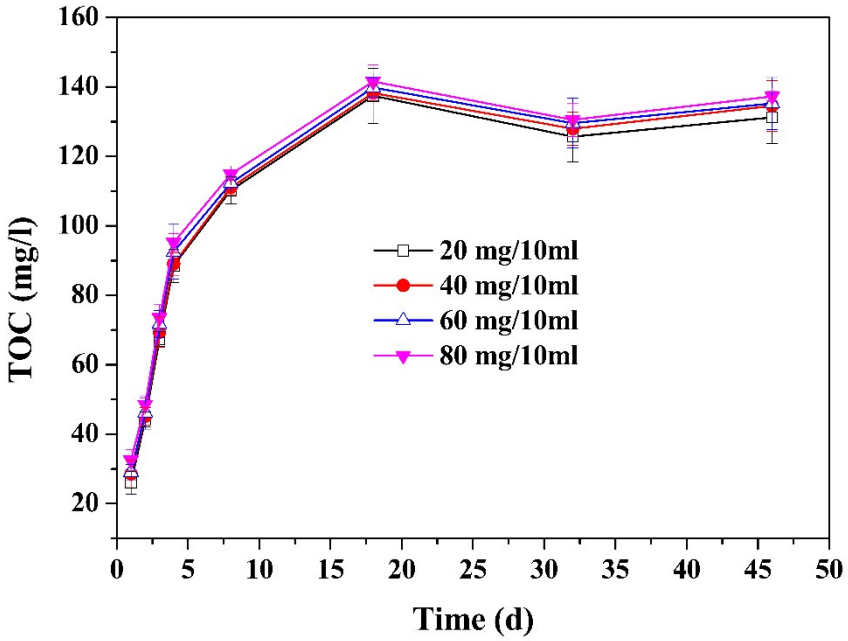

Fig. 3 TOC of lixivium of Semen litchi

3.4 Denitrification performances of Semen litchi

The denitrification performance of semen litchi was investigated and the results were presented in Fig. 4. Duiring the whole denitrification, the nitrate removal rate was between 98.8-99.5% and the nitrite concertration was lower than 0.01 mg/l. It was interesting to note that a high denitrification performance was achieved at the first day with no nitrite accumulation. That was indicated that semen litchi had short acclimation time[33] and the salinity did not had negatively effect on denitrification. Tab. 2 listed the comparison of denitrification rate of different solide carbon source, compared with the rice husk (90.6%-97.8%)[9], corncobs (90%)[1] and strach-PCL

193 (93.53-99.13%)[33] in fresh water, the denitrification rate of semen litchi was much
194 higher even in high salinity water, especially in the first day. The easily dissolved part
195 of semen litchi caused rapid microbial growth so that high removal rate of nitrate was
196 observed at the start-up. Then the starch and protein was gradually released out, and
197 nitrate was readily removed from the salinity water for a long time[1]. At last the nitrate
198 concentrations started to increase after a certain time because of the exhaustion of
199 carbon sources at which point the experiments were stopped.

200 The changes of COD and pH in effluent water were shown in Fig. 5. At the
201 beginning of this investigation, significant amounts of COD in effluent water were
202 observed because of the readily biodegradable organic matter in the semen litchi.
203 Days later, the concentration of COD decreased rapidly because the number of
204 microorganisms was increased and more carbon source was used. At last, the COD
205 concentration was too low to maintain the denitrification, so the nitrate concentration
206 increased[11]. In Fig. 5, the pH of the effluent was changed from 7.1 to 7.4, Although
207 the denitrification induced an increase in pH, the degradation in the semen litchi lead
208 to a decrease in pH[9]. So the pH of the effluent was maintained steadily during the
209 experiment.

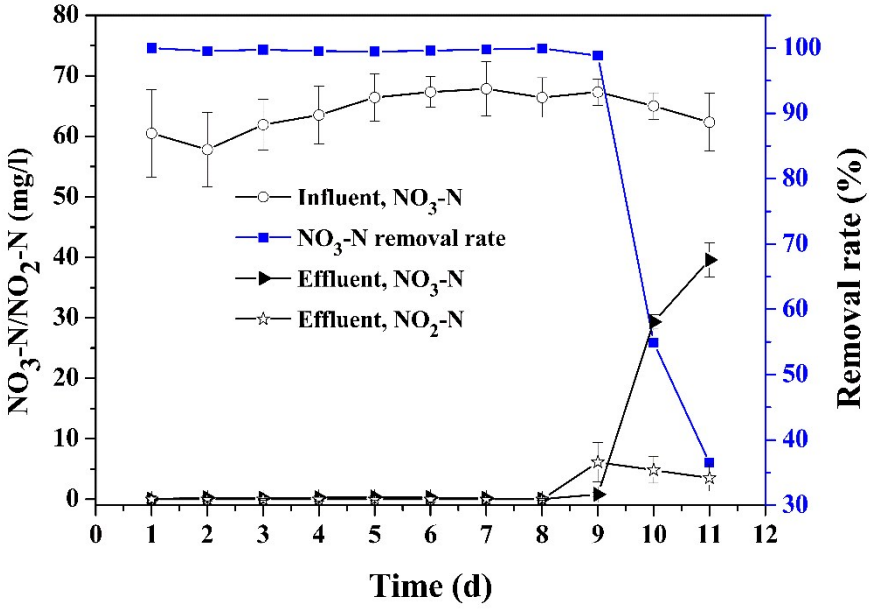

Fig. 4 Denitrification performance of Semen litchi

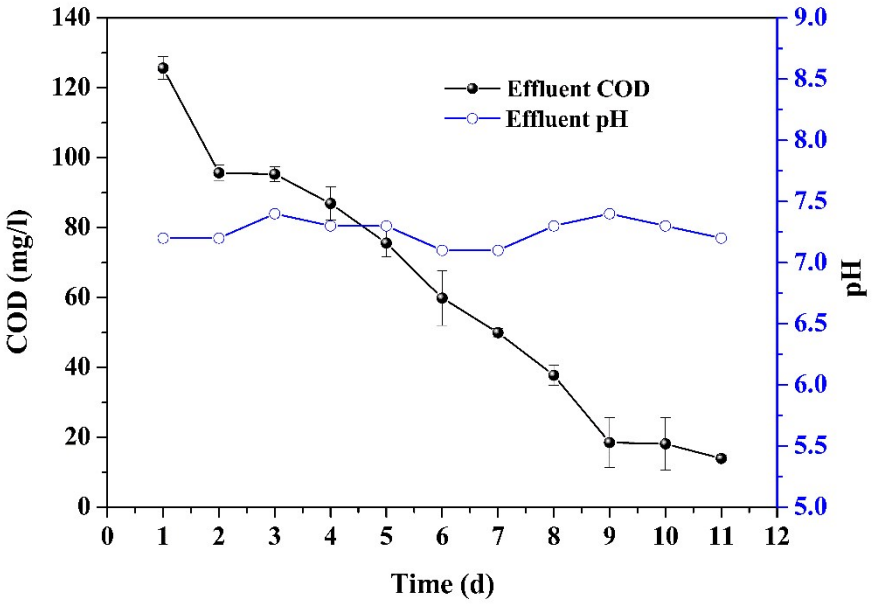

Fig. 5 The changes of pH and COD in effluent

The concentrations of NO_3-N, NO_2-N and COD variation with time were shown in Fig. 6. It was shown that nitrate concentration decreased quickly and the nitrate can be removed completely after 6 h. Nitrite concentration was increased in the first 2 h

and then decreased to 0.005 mg/l at 6 h. A high linear correlation between the concentration of NO_3-N and time was found and the degrees of correlation r^2 was 0.994, indicated that the denitrification process supported by semen litchi was a zero-order reaction[34,35]. The denitrification rate of semen litchi was calculated to be 192 mg N/(l d), which was much higher than the liquorice (6.2 mg N/(l d))[36], wheat straw (53 mg N/(l d)) and cotton (81 mg N/(l d))[9] (Tab. 2).

In Fig. 6, the concentration of COD in effluent water was remain stable at 10 mg/l (0-4 h) during the nitrate was removed completely stage. COD releasing was due to the biodegradation of semen litchi and the dissolved organics was served for microbes' growth and electron donor for denitrification. Once the releasing rate of COD exceeded the consuming COD in the denitrification process, COD was accumulated and increased for 6-12 h in the effluent[35].

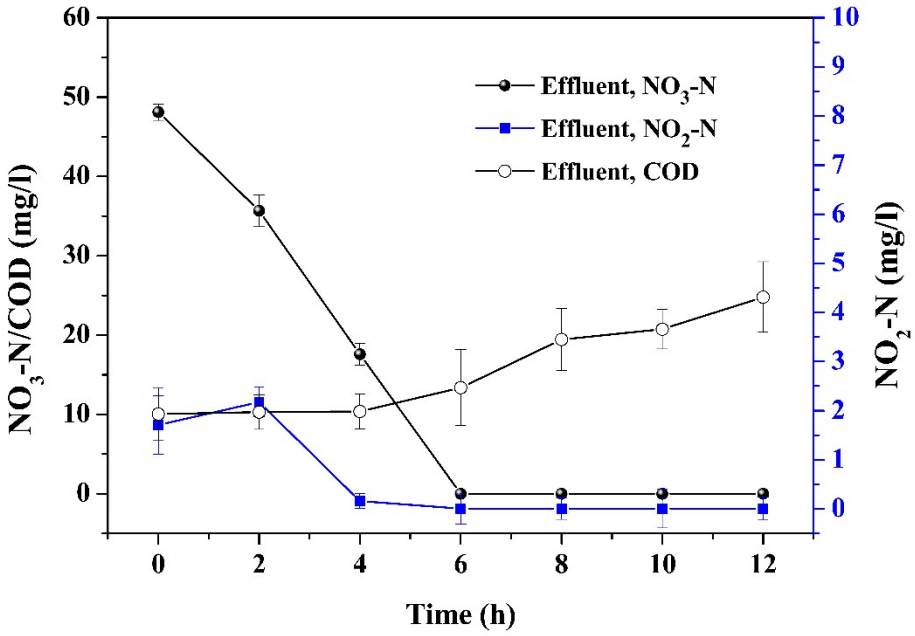

Fig. 6 The changes of NO_3-N, NO_2-N and COD in effluent

235 Tab. 2 Comparison of denitrification rate of different solide carbon source

Carbon source	nitrate removal rate	denitrification rate (mg N /(l d))	Reference
corncobs	90%	—	1
rice husk	90.6-97.8%	96	9
cotton	—	81	9
wheat straw	—	53	11
strach-PCL	93.5-99.1%	—	33
G. verrucosa	—	13	36
liquorice	—	6.2	36
giant reed	—	3.3	36
semen litchi	**98.8-99.5%**	**191**	this study

236 **3.5 DGGE analysis of microbial community**

237 The microbial community composition of the feed sludge and semen litchi
238 sludge were investigated through PCR-DGGE analysis. A total of 9 different bands in
239 the DGGE profile showed in Fig. 7 were excised from the gel and sequenced.
240 However, only the c, e bands were present in both lanes, indicated great changes in
241 microbial composition of semen litchi sludge. The GenBank closest relative matches
242 were detailed in Tab 3. It can be observed that when semen litchi was used as the
243 carbon source, *16S rRNA* gene sequences retrieved from DGGE were closely related
244 to representative of the *Sphingomonas* family (band b, d, f) and *Rhodospirillum*
245 family (band g). The species belonged or closely related to these microbial groups had
246 been reported as denitrifying bacteria[37,38,39]. In feed sludge, there were no excised
247 DGGE bands belonged to denitrifying bacteria, which may imply that the genera were
248 not the dominant members in feed sludge. Overall, results from DGGE analysis
249 demonstrated that the bacterial community in semen litchi sludge were highly affected
250 by using semen litchi as the carbon source. The increased biodiversity and
251 denitrifying bacteria in semen litchi sludge resulted in high denitrification rate and

rapid reaction rate in semen lithci reator.

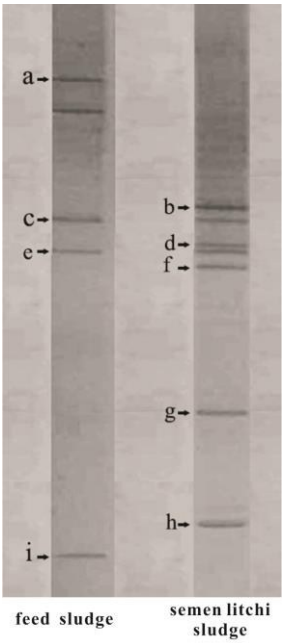

Fig. 7 DGGE profiles of PCR-amplified 16S rRNA gene fragments from feed sludge and semen litchi sludge.

Tab. 3 BLAST search results of sequences from DGGE bands

Band NO.	Accession number	Similarrity (%)	Closest relatives
a	NR_044490.1	99.42	*Butyricicoccus pullicaecorum*
b	NR_104893.1	94.07	*Sphingomonas paucimobilis*
c	NR_104695.1	99.26	*Clostridium cadaveris*
d	NR_116570.1	97.78	*Sphingomonas hankookensis*
e	NR_074264.1	98.52	*Caulobacter sp. K31*
f	NR_118263.1	99.26	*Sphingomonas sp. THG B283*
g	NR_074105.1	91.91	*Rhodospirillum centenum SW*
h	LN849621.1	100	*uncultured bacterium*
i	NR_074138.1	100	*Methylobacterium extorquens AM1*

4. Conclusions

In this paper, an agricultural waste semen litchi was used as the sole carbon source for biological denitrification in high salinity wastewater. The results showed that semen litchi had rough surface and gap structure, contained 48.8% strach and low levels of heavy metals. Duiring the whole denitrification processes, the nitrate removal rate of semen litchi could reach 98.8-99.5% and the nitrite concertration was

lower than 0.01 mg/l. The denitrification rate of semen litchi was 192 mg N/(l d) which was much higher than other agricultural wastes. Furthermore, salinity didn't have negative effect on denitrification. The DGGE analysis verified the biodiversity and denitrifying bacteria was increased after semen litchi was used as the carbon source. All the results were suggested that semen litchi was effective as the carbon source for the denitrifying microorganism. It could be used as an economical and environment-friendly carbon source for denitrification in high salinity wastewater or other low C/N wastewater. Moreover, it offered an alternative way to reuse agricultural waste.

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