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Immobilization of nitrite oxidizing bacteria using biopolymeric chitosan media

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Abstract

The effects of chitosan characteristics including the degree of deacetylation, molecular weight, particle size, pH pretreatment and immobilization time on the immobilization of nitrite-oxidizing bacteria (NOB) on biopolymeric chitosan were investigated. Nitrite removal efficiency of immobilized NOB depended on the degree of deacetylation, particle size, pH pretreatment on the surface of chitosan and immobilization time. Scanning electron microscope characterization illustrated that the number of NOB cells attached to the surface of chitosan increased with an increment of immobilization time. The optimal condition for NOB immobilization on chitosan was achieved during a 24-hr immobilization period using chitosan with the degree of deacetylation larger than 80% and various particle size ranges between 1–5 mm at pH 6.5. In general, the NOB immobilized on chitosan flakes has a high potential to remove excess nitrite from wastewater and aquaculture systems.

Key words: chitosan; immobilization; nitrite-oxidizing bacteria; pH adjustment

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Introduction

Over the last decade, outdoor-lining ponds have become an alternative aquaculture system instead of earthen ponds because of the low risk of water leakage and ease of cleaning after each crop. However, the accumulation of nitrogenous waste, especially nitrite which is commonly found in outdoor-lining ponds can directly harm aquatic animals and this is claimed to be a drawback of this lining pond system. To solve this problem, several techniques have been applied, for example, nitrogen uptake by phytoplankton (Chuntapa et al., 2003; Hargreaves, 2006; Lertsutthiwong et al., 2009) has been applied for nitrogen removal in aquaculture systems but planktonic algae cells are difficult to remove from aquaculture systems by conventional gravity or filtration. Consequently, the high density of algae in the pond also depletes dissolved oxygen in the pond at night time. Hence, the most common technique for nitrogenous waste removal is water exchange but it leads not only to an increase in production costs and the risk of pathogen outbreak but also to water discharge polluting the water resources. Among several techniques applied for nitrogen removal from aquaculture systems (Crab et al., 2007; De Schryver et al., 2008; De Schryver and Verstraete, 2009; Fierro et al., 2008; Liu et al., 2000; Ruiz et al., 2006), biofiltration using nitrification process is accepted as the most feasible nitrogen treatment process.

Biofiltration or the conversion of ammonia to nitrate by nitrifying bacteria is one of the most efficient processes for nitrogen treatment in recirculating aquaculture systems (RAS). Nitrification takes place with two groups of autotrophic bacteria, ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), both of which comprise slow-growing species and are highly sensitive to the changing environment. Setting up a new RAS requires a long bacteria acclimation period, e.g. 3–4 weeks, until both the AOB and NOB are fully activated (Shan and Obbard, 2001). Without complete acclimation, an accumulation of highly toxic nitrite is generally found due to the incomplete nitrification process. Biofiltration in the RAS represents an immobilization of AOB and NOB on a certain media in order to maintain the water quality in aquaculture system (Dong et al., 2011; Manju et al., 2009; Sumino et al., 1992). Moreover, most of the biofilter devices are applied to indoor RAS such as aquariums or indoor intensive

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fish tanks. Conventional biofiltration RAS using nitrifying bacteria immobilized on plastic or other materials such as ceramic or beads is not applicable for outdoor lining-ponds which are generally large (up to 1 Hectare) and exposed to direct sunlight. Other immobilization techniques based on microbial cell entrapment were also reported. For example, poly(vinyl alcohol) matrices in the forms of particle (Rostron et al., 2001), cryogel (Lozinsky and Plieva, 1998) and hydrogel (Nishio et al., 1998) were developed using freeze-thawing technique. Although poly(vinyl alcohol) matrices performed good cell immobilization, but the preparation by freeze-thawing technique is quite expensive and may danger to some bacteria that sensitive to temperature (Lozinsky and Plieva, 1998). Moreover, these entrapment cells are not suitable for the mass production. Hence, this study proposes the use of nitrifying bacterial immobilizing on biopolymeric materials as an alternative biofilter for large aquaculture ponds.

Biopolymeric chitosan or poly [β-(1→4)-2-amino-2-deoxy-D-glucopyranose] is mainly extracted from shrimp biowaste (Lertsutthiwong et al., 2002). It is composed of *N*-acetyl-D-glucosamine and D-glucosamine units linked by β-(1→4)-glycosidic bonds as shown in **Fig. 1**. Due to its properties such as biodegradability, biocompatibility, cationic character and non-toxicity, chitosan can be used in various applications including water and wastewater treatments (Franco and Peter, 2011; Rinaudo, 2006). For example, chitosan can form complexes with microorganism (El-Mamouni et al., 1998; Strand et al., 2003) due to its cationic character. Various studies have focused on flocculation and the immobilization of microorganisms (Fierro et al., 2008; Kaseamchochoung et al., 2006; Lertsittichai et al., 2007; Lertsutthiwong et al., 2009; Wang et al., 2008), but the use of chitosan as a biofilter media for NOB immobilization has not been evaluated. Therefore, the present work was undertaken to optimize immobilization conditions by investigating the effects of chitosan characteristics, pH pretreatment and the immobilization time on the nitrite removal rate of NOB immobilized chitosan. The chitosan with attached nitrifying bacteria is therefore a novel practical technique for water quality control in outdoor-lining aquaculture ponds because it can be directly added into the pond to enhance nitrification process so the toxic ammonia and nitrite during the first month of the crop could be controlled.

1 Materials and method

1.1 Chitosan

Chitosan with a degree of deacetylation (DD) of 91% and molecular weight of 410,000 g/mol was supplied by Ebase (Bangkok, Thailand). Chitosan with molecular weight of 310,000 and 580,000 g/mol and chitin were supplied by A.N. Laboratory (Samut Sakorn, Thailand). The DD of these chitosans was controlled at 85% ± 5%. Chitosan with a DD of 92% having molecular weight of 200,000 g/mol was prepared by alkaline deacetylation of shrimp chitin (Lertsutthiwong et al., 2002). Prior to use, the chitosan flakes were sterilized with 70% ethanol for 2 min and their surface charges modified by soaking with buffer at the required pH.

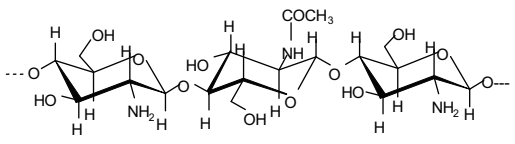

Fig. 1 Chemical structure of chitosan.

1.2 Nitrifying bacteria

The mix-culture of nitrifying bacteria naturally attached as biofilm on polyethylene media (BCN-009, 2H GmbH, Germany) was collected from an indoor recirculating shrimp tank with 30 PSU salinity at the Center of Excellence for Marine Biotechnology, Chulalongkorn University in Bangkok, Thailand. This media was porous plastic with a 10 mm diameter, 8 mm in height and 864 m^2/m^3 in specific surface area. Incubation of the NOB in the laboratory at 28 ± 3°C was performed by mixing the biofilter media from the shrimp tank with the new filter media in 30 PSU seawater. The new biofilter media was disinfected with chlorine before being incubated with natural nitrifying bacteria population under selective conditions for NOB in which 10 mg-N/L sodium nitrite was added as the sole nitrogen source. Alkalinity in the water was maintained between 100–120 mg/L and oxygen was continuously supplied to the system by aeration.

1.3 Immobilization of NOB on chitosan

Twenty pieces of plastic biofilter media (BCN-009) with active NOB biofilm were transferred into a container containing 40 mL of sterilized 30 PSU seawater with 2 mg-N/L nitrite and sonicated for 4 min to the detached NOB cells. Thereafter, the biofilter media were replaced with 0.60 g of sterilized chitosan flakes under 150 r/min agitation at 25°C. With this immobilization process, a significant number of bacterial cells could be transferred from the biofilter media to the chitosan flakes. The chitosan agitation period was varied at 3, 6, 12 and 24 hr. The efficiency of NOB immobilized chitosan on nitrite removal was determined by placing 0.60 g of NOB immobilized chitosan flakes in 40 mL seawater containing 2 mg N/L of nitrite. The decrease in nitrite concentration was monitored using the standard method of seawater analysis (Strickland and Parson, 1972). Moreover, bacterial attachment on the surface of the chitosan flakes was visualized by scanning electron microscope (SEM).

To study the effect of pH pretreatment on NOB immobilization, the sterilized chitosan was soaked in a buffer solution at pH 5.5, 6.5 or 7.5 for 2 min before immobilizing with NOB as mentioned above. Nitrite removal rates were then evaluated to find an appropriate condition for bacterial

immobilization. After achieving a suitable condition, the effect of DD (i.e. 82% and 91%), molecular weight (i.e. 200,000, 410,000 and 580,000 g/mol) and size of chitosan (i.e. 1–2, 3–5 and 8–10 mm) were then investigated.

1.4 Statistical analysis

All of the tests were performed in triplicate and data was expressed as mean values ± standard deviation. Statistical analysis was performed by analysis of variance with a further Duncan's multiple range test.

2 Results and discussion

Mixed culture of nitrifying bacteria from RAS biofilters was immobilized on plastic media after 45 days incubation in shrimp culture tanks. This was following by NOB selection in nitrite broth under laboratory conditions. The desired condition for NOB selection is presented in **Table 1** (Hart and O'sullivan, 1993; Lawson, 1995; Satoh et al., 2000; Timmons and Losordo, 1994). The activity of selected NOB on plastic media is shown in **Fig. 2**. It was found that nitrite concentration in the water was reduced to 0.20 mg-N/L within 8 hr and nitrite removal rate was about 28.09 ± 0.58 mg-N/(m^2·day). Consequently, nitrate concentration in the test chamber increased in proportion to the decrease of nitrite while ammonia remained constant. This indicated the complete nitrite oxidation process.

2.1 Effect of immobilization time

The results in **Fig. 3** illustrate that the variation of immobilization time influenced the nitrite removal efficiency of the NOB immobilized chitosan. The reduction of nitrite concentration was a function of incubation time (**Fig. 3a**). A longer immobilization time exhibited a faster nitrite removal rate (**Fig. 3b**). As expected, a greater immobilization time allowed more NOB cells to be attached to the surface of chitosan. This was confirmed by SEM analysis as shown in **Fig. 4**. It was clear that a higher number of NOB were found on the chitosan surface after 24 hr immobilization time, hence a higher nitrite removal rate was the result of cell coverage on chitosan surface.

Table 1 Quality of nitrite broth used for selection of nitrite-oxidizing bacteria (NOB)

Parameter	Value
Alkalinity (mg/L)	113.33 ± 8.16
Salinity (PSU)	30.33 ± 0.52
Temperature (°C)	26.50 ± 0.15
Dissolved oxygen (mg/L)	5.43 ± 0.21
pH	7.34 ± 0.18

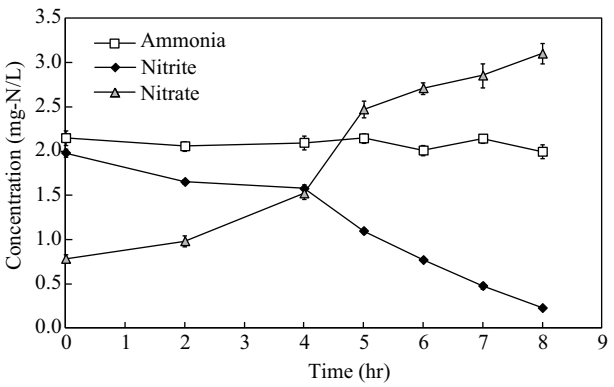

Fig. 2 Activity of selected NOB stock culture in terms of ammonia, nitrite and nitrate concentrations.

In general, the incubation period of NOB on conventional nitrification biofilter media takes about 30–45 days because of the limitation of NOB cells in natural waters and the substantially lower growth rate of the nitrifying bacteria (Ruiz et al., 2006). According to the results of this study (**Fig. 3**), the use of chitosan as a biofilter media can reduce the immobilization time by the immediate transfer of NOB from pre-acclimated stock of NOB into the chitosan flakes. This phenomenon might be explained by the cationic character of chitosan in slightly acidic conditions. In the beginning, sterilized chitosan modified its surface through soaking in buffer at pH 6.5 that resulted in high cationic charges on the chitosan surface. Consequently, this treated chitosan could interact with the anionic charges of the bacterial cell wall (Lertsutthiwong et al., 2009; Strand et al., 2003). On the other hand, conventional plastic biofilter media are non-ionic polymers, so the attachment of NOB to plastic surfaces without electrostatic interaction requires a longer time with, probably, a lower in adhesion strength.

2.2 Effect of pH pretreatment

As expected, the nitrite removal efficiency of NOB immobilized on chitosan strongly depended on the pH pretreatment of the chitosan surface (**Fig. 5**). Compared with the control (without pH adjustment), pH adjustment between 5.5 to 7.5 significantly affected nitrite removal of the immobilized NOB on chitosan. Statistical analysis revealed that the pH adjustment to acidic (pH 5.5–6.5) could enhance the NOB attachment to the chitosan surface and, hence, nitrite removal was significantly enhanced. This might be explained by the fact that more protons are available at lower pH and resulting in more positive charge on $–NH_2$ on the chitosan molecules (Chatterjee et al., 2007). In the other words, the charge density of chitosan depends on pH pretreatment (Kasemchochoung et al., 2006). With a pH of less than 7, chitosan contains high cationic charges which allow ionic bonding to anionic cell wall of the bacteria. In practice, chitosan surface should be treated with the buffer at pH 6.5 before the NOB immobilization process.

2.3 Effect of particle size of chitosan

Figure 6 illustrates the reduction of nitrite concentration as a function of the particle size of chitosan. NOB immobilized on chitosan with a size of 1–2 mm and 3–5 mm

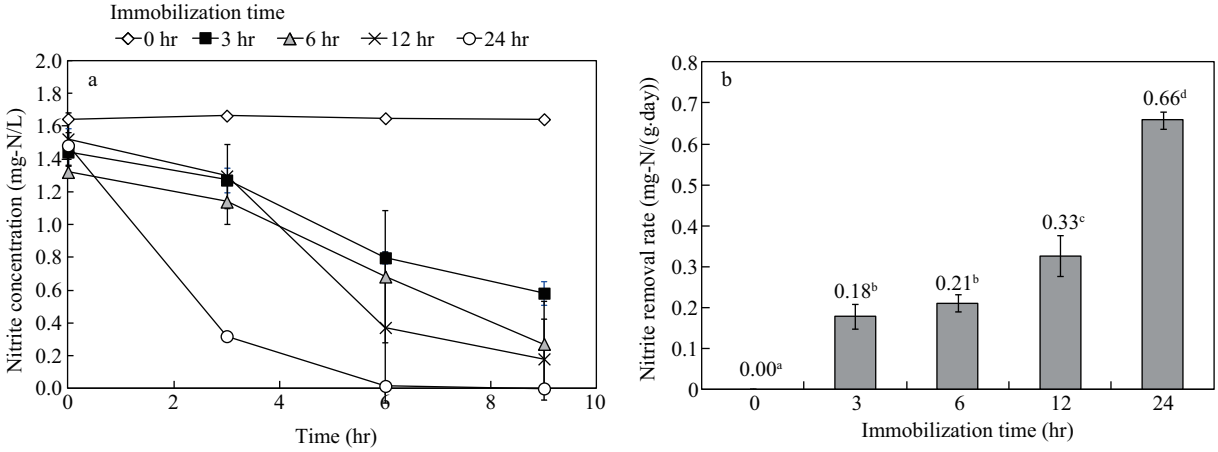

Fig. 3 Effect of immobilization time on nitrite concentration (a) and nitrite removal rate (b). The different letters in a graph under each immobilization time represent a significant difference ($P < 0.05$).

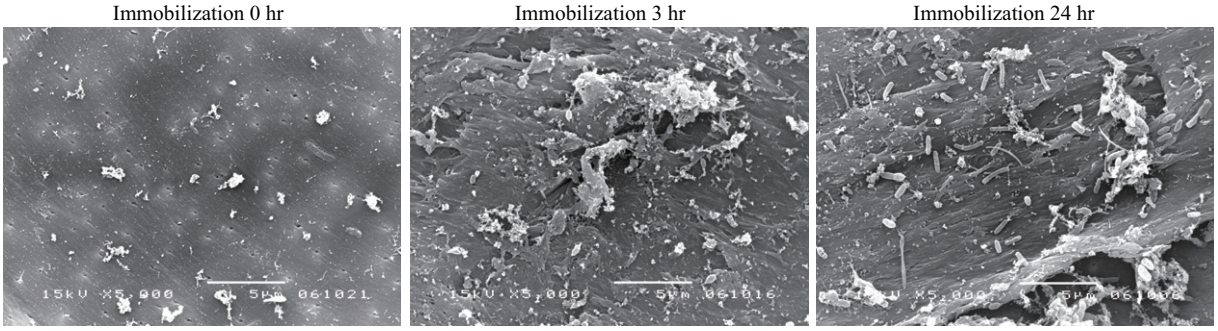

Fig. 4 SEM images of bacteria immobilizing on chitosan after immobilization for 0, 3 and 24 hr.

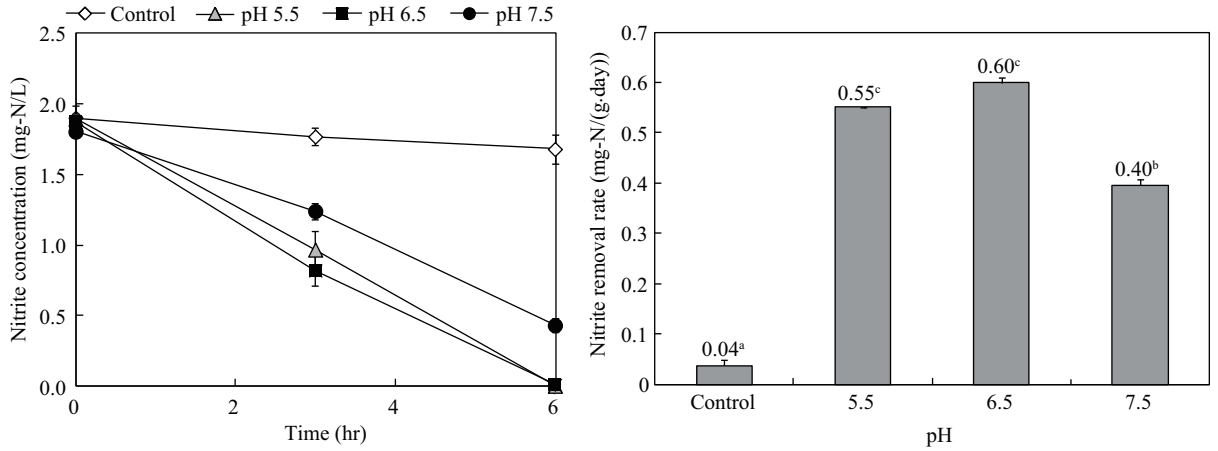

Fig. 5 Effect of pH pretreatment on nitrite concentration and nitrite removal rate. The different letters in a graph represent a significant difference ($P < 0.05$).

could reduce almost 100% of nitrite concentration within 2.5 hr (**Fig. 6a**) with a nitrite reduction rate of 1.18 ± 0.03 and 1.12 ± 0.05 mg-N/(g·day) (**Fig. 6b**), respectively. However, chitosan with a size of 8–10 mm took about 3 hr to remove nitrite with a rate of 0.84 ± 0.03 mg-N/(g·day). Similar results were also observed in nitrifying bacteria immobilized on wood particles reported by Manju et al. (2009). Wood particles with a size of 0.3–1.5 mm gave the best efficiency of nitrite removal. However, statistical analysis did not show a significant difference between chitosan with a size of 1–2 mm and 3–5 mm ($P > 0.05$) but these two particle sizes showed significant difference from those sized 8–10 mm ($P < 0.05$). According to the results, the optimal size of chitosan used for the immobilization of NOB should be lower than 5 mm.

2.4 Effect of degree of deacetylation of chitosan

It was clear that deacetylation enhanced the NOB attachment to the chitosan surface. **Figure 7** demonstrates that NOB immobilized on chitosan with either DD 82% or DD

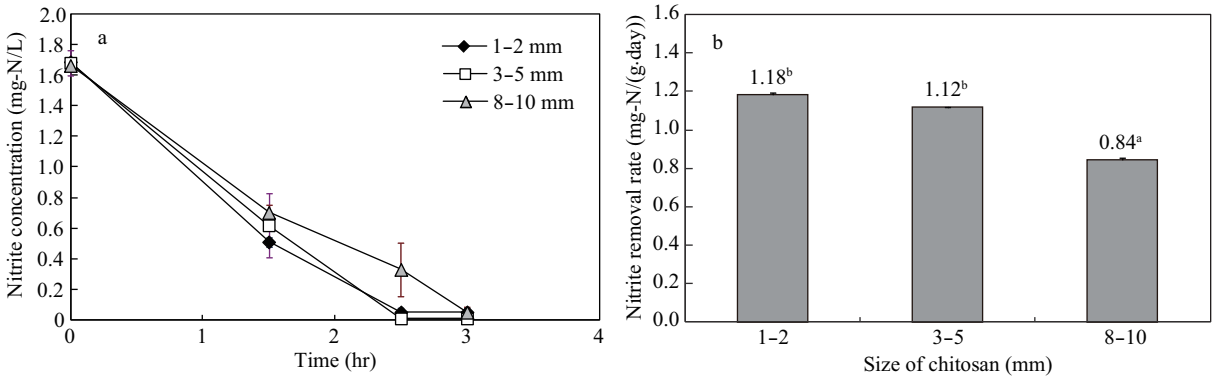

Fig. 6 Effect of particle size of chitosan on nitrite concentration (a) and nitrite removal rate (b). The different letters in a graph represent a significant difference ($P < 0.05$).

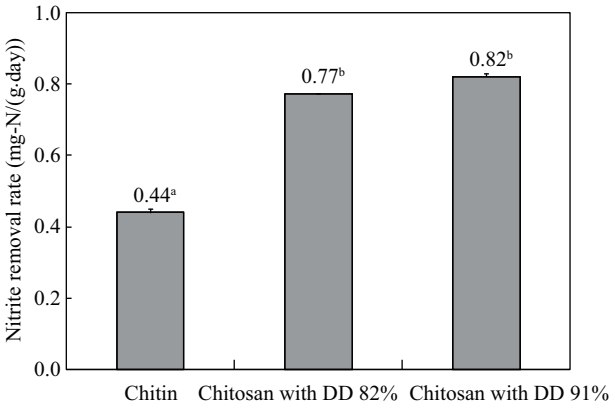

Fig. 7 Effect of degree of deacetylation of chitosan on nitrite removal rate. The different letters in a graph represent a significant difference ($P < 0.05$). DD: degree of deacetylation.

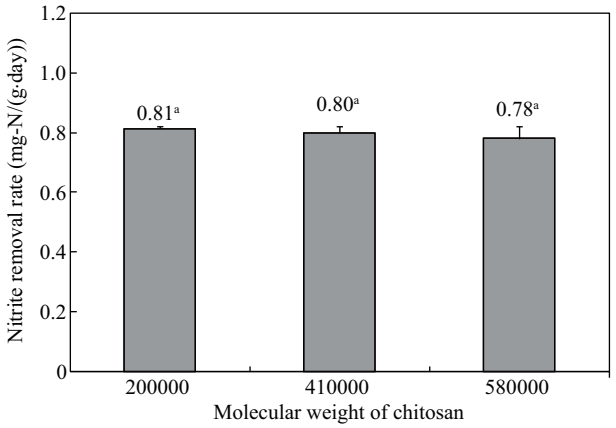

Fig. 8 Effect of molecular weight of chitosan on nitrite removal rate. The letters in a graph represent no significant difference ($P > 0.05$).

91% (molecular weight of 310,000–410,000 g/mol) had a significantly higher nitrite removal rate compared to that of chitin. For example, NOB immobilized on chitosan with a DD of 91% could remove nitrite at a rate of 0.82 ± 0.05 mg-N/(g·day) whereas NOB immobilized on chitin only managed 0.44 ± 0.03 mg-N/(g·day). From the statistical analysis, chitosan with DD of 82% and 91% did not have a significant effect on nitrite removal rate ($P > 0.05$). In other words, chitosan used as a biofilter should contain DD at above 80%. This observation can be explained by charge density. After adjusting the surface of chitosan with a buffer at pH 6.5, chitosan with a DD above 80% contains higher cationic charges than chitin which has DD 10%–15% (Lertsutthiwong et al., 2002). This results in more ionic bonding with the anionic surface of the bacterial cell wall.

2.5 Effect of molecular weight of chitosan

As shown in **Fig. 8**, the molecular weight of chitosan between 200,000–600,000 g/mol (controlled DD of 85% ± 5%) gave a similar nitrite removal rate of approximately 0.80 mg-N/(g·day) ($P > 0.05$). It may be concluded that the molecular weight of chitosan did not affect the nitrite removal efficiency of NOB immobilized on chitosan whereas the degree of deacetylation had a significant effect on the nitrite removal rate as discussed above.

3 Conclusions

Chitosan is a promising biopolymer that can be used as an alternative biofilter media for nitrite oxidizing bacteria (NOB). The immobilization of NOB on the surface of chitosan flakes depends on immobilization time, pH pretreatment, particle size and degree of the deacetylation of chitosan. On the other hand, the molecular weight of chitosan did not affect nitrite removal efficiency. With this study, the optimal immobilizing process was the use of chitosan flakes with a size between 1–5 mm with a degree of deacetylation higher than 80% and the chitosan pretreated with a buffer at pH 6.5 to modify surface charges. An optimal immobilization period of 24 hr is required. The NOB immobilized on chitosan flakes has a high potential to remove excess nitrite from wastewater and aquaculture ponds.

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