

Effect of different concentration of profitable *Bacillus* on bioremediation of common carp (*Cyprinus carpio*) pond discharge

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Abstract

A 4 days study was conducted to determine the effects of Different concentration of bioaugmentor bacterial strain *Bacillus licheniformis*, *B. subtilis*, *B. polymyxa*, *B. laterosporus* and *B. circulans* (Protexin Aquatech, UK) on adjustment of parameters of water quality as TAN, NO₂-N, NO₃-N and turbidity from the effluent of Common Carp ponds. Effects of time and concentration were studied as a completely randomized split-plot design. Time and concentration and their interaction had a significant difference ($P < 0.05$) on changes of total ammonium nitrogen, nitrate nitrogen, nitrite nitrogen and turbidity. The total counts of bacteria recorded in the water of bioremediated tanks were also lower than that in the control tank.

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Introduction

During the past twenty years, aquaculture and mariculture have significantly improved. However, the effluent of aquaculture activity without treatment procedure increases pollution of environmental acceptor water and damages the ecology of culture areas. This is a result of aquaculture wastewater study, which contains significant amounts of nutrients such as nitrogen, phosphorus and the amount of organic material reduces the water quality and can be a potential condition for pathogenic microorganisms quickly grow and distribute (Zhou, L.i, Jun & Bo 2009). Efficient use and reuse of water resources requires affordable and efficient strategies which help to reduce pollution of wastewater. Chemical and biological treatment process are two main

methods during the treatment of wastewater. Chemical treatment are more effective for the removal of non-biodegradable materials than the biological methods but the disadvantages of chemical treatment are massive (Makridis, Bergh, Skjermo & Vadstein 2001; Jafaryan, Soltani, Noferesti & Ebrahimi 2011), thus biological treatment of wastewater is Supported in the last few decades (Akpore & Muchie 2010).

In recent decades bioremediation by probiotics has become highly regarded and many researchers around the world are engaged in research and study associated with the influence of useful bacteria on treatment contaminated water and reduction of pollutants of water source. In recent years, beneficial results and experiences have been obtained from these studies (Boyd, Hollerman, Plumb & Saeed 1984). Bioremediation is a term that is used to improve water quality in aquaculture by performance of probiotics. Treatment with the help of probiotics as a biological control method is considered. biocontrol is related to the elimination of waste like parasites or specific pathogens (Moriarty 1998). Moriarty (1998) proposed the microbial probiotics as water additives. Burford, Thompson, McIntosh, Bauman & Pearson (2003); Devaraja, Yusoff & Shariff (2002); Queiroz & Boyd (1998); Vezzulli, Pruzzo & Fabiano (2004) reported that the results of bioremediation experiments showed that elimination of organic pollutants

can be done with microbial products and promote water quality.

According to the published findings, application of beneficial bacteria in the water of fish pond is based on two principles: biocontrol with the goal of being antagonistic to pathogens (Gatesoupe 1999; Moriarty 1998; Nogami & Maeda 1992; Skjermo & Vadstein 1999; Rengpipat, Phianphak, Piyatiratitivorakul & Menasveta 1998). Especially bacteria found in companies, as biocontrol agents appears worthwhile instead of the negative effects of antibiotics (Abraham, Shanmugham, Uma, Palaniappan & Dhevendaran 2001). Bioremediation for controlling water quality (van Rijn, Fonarev & Berkowitz 1995; van Rijn & Nussinovitch 1997; Prabhu, Nazar, Rajagopal & Khan 1999; Queiroz & Boyd 1998) that Singh & Radhika (2001) said. Most probiotics used in aquaculture as biological control agents belong to the Lactic Acid Bacteria (*Lactobacillus*, *Carnobacterium* etc.), *Vibrio* (*Vibrio alginolyticus*), *Bacillus*, and *Pseudomonas*.

The main sources of nitrogenous substances in rearing fish culture are fish excretion and the matter that is released from the sediments during the conversion of organic matter into inorganic substances, although nitrogen fixation by cyanobacteria and atmospheric diffusion are important (Ayyappan & Mishra 2003). Total ammonia nitrogen (TAN = $\text{NH}_3\text{-N} + \text{NH}_4\text{-N}$) removal in a recirculating aquaculture is very important because of $\text{NH}_3\text{-N}$

is toxic even at low concentrations (Eding, Kamstra, Verreth, Huisman & Klapwijk 2006). Nitrogenous substances contained in sewage discharges have the important role in eutrophication of receiving waters and one of the most economical processes for nitrogen removal from sewage is a biological nitrification–denitrification (Gupta & Gupta 2001).

Mevel and Prieur (Mevel & Prieur 2000) reported that *Bacillus* strains are known to be involved in heterotrophic nitrification. *Bacillus* sp. is one of the popular microbial products for aquaculture, in the Chinese market Liu & Han (2004) stated the popularity of *Bacillus* sp is due to its established manufactory technique, mass supply, simple preservation and convenient transportation. The role of *Bacillus* sp. due to its adaptation in aquatic environments is very important. *Bacillus* is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum Firmicutes, spore forming bacteria, used generally as a probiotic due to its high resistance against extreme environmental conditions and also its high reproducibility and low cost of increased production is considered a beneficial microorganism than others (Wang, Li & Lin 2008).

Moriarty (1998) stated that controlled species of *Vibrio luminous*, by adding many strains of *Bacillus* in the shrimp ponds (Penaeidae), finally increased shrimp survival because of the inhibitory activity of *Bacillus* against the *Vibrio luminous* in the Ponds. shrimp

survival due to a direct effect of *Bacillus* performance on animal health and decomposing organic matter and promote water quality. In two different investigations, Jafaryan *et al* (2011) found that adding probiotics *Bacillus* in rearing water of *Cyprinus carpio* and *Ctenopharyngodon idella* larvae enhanced the growth of the fish.

Ghosh, Sinha & Sahu (2008) stated that the use of *Bacillus subtilis* in the rearing water of the livebearing fishes resulted in improvement of water quality. Use of *Bacillus subtilis* in the rearing water of the livebearing fishes resulted in improvement of water quality organic matter and nitrogen removal from reclaimed wastewater used as landscape water was carried out by Zhao, Hu, Chen, Zha & Liang (2009) by using the *Bacillus cereus*.

This study was designed to investigate the effects of different concentrations of probiotics at different times on bioremediation. This article tries to find the best time and concentration of probiotics on adjustment of water quality parameters in rearing tanks as the living environment of reared fish, bioremediation of effluent, re-use of water resources, to prevent pollution of water acceptor and to help preserve valuable natural environmental organisms.

Material and Method

Experimental Materials

The probiotic *Bacillus* was prepared from Protexin Co (Iran-Nikotak). The blends of

probiotic bacilli (*Bacillus licheniformis*, *B. subtilis*, *B. polymyxa*, *B. laterosporus* and *B. circulans*) from suspension were provided. Three concentrations of bacterial suspension, 1×10^6 , 1×10^7 , 1×10^8 CFU/L was determined by optical density at 610 nm in a spectrophotometer and the colony forming unit (CFU) of probiotic bacilli were tested by microbial culture in Tryptic Soy Agar (TSA). The effluent prepared from cultivation system of Common Carp (*Cyprinus carpio*) in fishery lab of University of Gonbad kavos, Iran.

Experimental Setup

This experiment was conducted in 4 treatments, each with three replicates. 12 plastic tanks with a volume of 3 liters of wastewater was prepared. Treatment 2, 3 and 4 incubated by the blend of bacilli were added directly to at a concentration of 1×10^6 , 1×10^7 and 1×10^8 CFU/ liter, respectively and in control treatment (T-1) didn't add any bacilli. All of trial tanks were aerated water temperature was 21-23 °C.

Measured parameters

At the starting of the experiment and after inoculating of bacteria into the waste water, the changes of water quality parameters such as TAN, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$ and turbidity was measured at intervals of 24, 48 and 72 h. $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, TAN by spectrophotometer Manufacturing CoHANNA (HI83200 Model). turbidity with a portable multi-line to be measured after calibration.

Total count of bacteria in water

Every day, water samples were collected in sterile containers, serially diluted and the total bacterial counts were determined by spread plating in triplicate on TSA with incubation at 30°C for 48 h (Ghosh *et al.* 2008).

Data Analysis

Analyzed in a completely randomized split-plot design using MSTATC statistical software to compare LSD procedure was performed at the 5% level. Before analysis, data normality was tested. the results of the total counts of bacteria were processed by log10 transformation.

Result

The parameters of water quality are presented in Table 1. The results show the time and concentrations and their interaction effect on $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, TAN turbidity and total count of bacteria. The different concentrations (0, 10^6 , 10^7 , 10^8 cfu/l) are indicated by 1,2,3,4 and different times (0, 24, 48, 72) are introduced by 1, 2, 3, 4.

Significant difference was observed for these parameters between the treatment groups. The highest TAN, lowest $\text{NO}_3\text{-N}$ was obtained in control treatment T1at the first day and the lower TAN, highest $\text{NO}_3\text{-N}$ was observed in experimental treatment T4 after 72 h (Table2). The highest turbidity was obtained in control treatment T1at the first day and after 72 h was not significantly difference between 2, 3 and 4 treatment.

Table 1 Variance analysis: the effect of time, concentration and their interaction on NO₂-N, NO₃-N, TAN and turbidity

Source	df	NO ₂ -N	NO ₃ -N	TAN	Turbidity	Total count colony
time	3	1.52**	4.38**	7.042**	6.24**	5.14**
r ×time	8	0.009	0.16	0.01	0.047	0.23
Concentration	3	0.04**	1.24**	0.66**	1.37**	0.67**
Time×con	9	0.021**	1.62**	0.01**	0.21**	0.2**
r ×time×con	24	0.006	0.2	0.007	0.034	0.076
Coefficient of Variance		11.34	8.45	4.56	3.38	4.07

Significant difference at level 0.01 indicated by **

Table 2 Changes in the water quality parameters. The different concentrations (0, 10⁶, 10⁷, 10⁸ cfu/l) are indicated by 1,2,3,4 and different times (0, 24, 48, 72) are introduced by 1, 2, 3, 4.

time	Conc.	NO ₂ -N (mg/l)	NO ₃ -N (mg/l)	TAN (mg/l)	Turbidity (NTU)	Total count CFU/ml
1	1	0.28±0.006f	5.26±0.25de	2.58±0.047a	6.4±0.14a	7.53±0.41a
1	2	0.28±0.006f	5.26±0.25de	2.58±0.047a	6.4±0.14a	7.53±0.41a
1	3	0.28±0.006f	5.26±0.25de	2.58±0.047a	6.4±0.14a	7.53±0.41a
1	4	0.28±0.006f	5.26±0.25de	2.58±0.047a	6.4±0.14a	7.53±0.41a
2	1	0.77±0.14c	4.9±0.26ef	2.42±0.07ab	5.9±0.3b	6.89±0.25b
2	2	0.58±0.06de	4.36±0.51f	2.07±0.15cde	4.9±0.18de	6.54±0.6bcd
2	3	0.52±0.042e	4.36±0.20f	1.73±0.15bcd	5.2±0.20cd	6.37±0.32cd
2	4	0.49±0.097e	4.73±0.40ef	1.99±0.11bc	4.6±0.05ef	6.65±0.37bcd
3	1	0.73±0.046c	5.36±0.20cde	2.06±0.09def	6.3±0.15a	7.54±0.31a
3	2	0.71±0.046cd	6.06±0.51bc	1.31±0.1defg	5.5±0.15c	6.91±0.13b
3	3	0.55±0.034e	5.83±0.20cd	1.23±0.07efgh	5.2±0.33cd	6.79±0.13bc
3	4	0.74±0.063c	5.43±0.15cde	1.16±0.07def	5.3±0.11c	6.71±0.31bcd
4	1	1.12±0.030b	4.43±0.15f	1.40±0.04fgh	5.2±0.52cd	6.32±0.28d
4	2	1.29±0.17a	5.3±1.1de	0.86±0.06fgh	4.4±0.76f	6.45±0.16bcd
4	3	1.05±0.16b	6.8±0.50ab	0.9±0.1h	4.5±0.71f	5.48±0.22e
4	4	1.11±0.063b	7.3±0.36a	0.7±0.04 g	4.5±0.78f	5.63±0.28e

Values are means ± SD of treatment groups. Values in the same column with different superscripts for each species are significantly different (P<0.05).

Discussion

In the natural aquatic environment, energy and carbon source were provided for survival and growth of Microorganisms using the

contaminant, thus they play important roles in the geological cycle of elements and

transformation of natural chemicals (Watanabe 2002).

The result showed that administration of the *Bacillus sp.* via direct inoculation to effluent rearing tanks the result showed that administration of the *Bacillus sp.* via direct inoculation to effluent rearing tanks significantly decreased total ammonia nitrogen (TAN = $\text{NH}_3\text{-N} + \text{NH}_4\text{-N}$) in bacterial treatment compared with blank treatment but $\text{NO}_3\text{-N}$ significantly increased in experimental treatment. Koops and Moller (1992) presented Bacilli contribute to nitrification in aquatic systems and utilize ammonium ion as the nitrogen source for its growth under aerobic conditions. *B. subtilis* was involved largely in nitrification (Kim, Joo Park, Sook Cho, Nam, Park & Bajpai 2005) and Ghosh *et al* (2008) inoculated *B. subtilis* in rearing tanks of ornamental fish and reported heterotrophic Bacilli was involved in the nitrification process and converted organic matter such as excreta of fish, remaining food substance to nitrate and phosphate and increased nitrate levels in the bioremediated tanks. Kim, Joo Park, Sook Cho, Nam, Park & Bajpai (2005); Queiroz & Boyd (1998) and Prabhu, Nazar, Rajagopal & Khan (1999), according to their findings, reported decreased ammonia levels, converting it to nitrate by using bioremediators. The metabolic pathways of heterotrophic bacillus strains were less complex than autotrophs (Kim *et al.* 2005) and *Bacillus* species could accomplish anaerobic dissimilatory reduction of

nitrate to ammonia via nitrite (Tiedje 1988). Jafaryan *et al.* (2011) indicated that the addition of probiotic bacilli at level $1 \times 10^6 \text{ CFU.L}^{-1}$ to rearing tanks water had positive effects on the growth parameters of grass carp and might be due either to an effect on animal health or improved water quality by their action. In contrast with our results Boyd *et al.* (1984) reported that the addition of commercial probiotic bacteria had no significant effect on water quality parameters. Sharma & Bukhar (2000) indicated that the addition of Aquazyn-TM-1000, a probiotic, had no significant difference on the water quality of *Cyprinus carpio* var. *communis*.

Water turbidity decreased. The water transparency was also improved by macromolecular degradation of microorganisms and improved by both the decomposition and natural sedimentation of *B. cereus* (Zhao *et al.* 2009).

Investigation of bacterial total count in water showed that the total bacteria in experimental treatment significantly decreased compared to blank treatment. The reason for this was the competition between bacterial flora and inoculated *Bacillus sp.* (Zhao *et al.* 2009). Moriarty (1998) and Verschuere, Rombaut, Sorgeloos & verstraete (2000) suggested that bacilli are able to use nutrients and space more than other bacteria and out-compete them by making antibiotics. Bacilli spatter many exoenzymes that can reduce slime and biofilms

around gram-negative bacteria and influence via this layer (Zhao *et al.* 2009) Finally. the population of gram-negative bacteria decreases. Ali (2000) and Makridis *et al.* (2001) encountered lower counts of *Aeromonas* and other gram-negative bacteria in water of fish cultured by administration of probiotic bacterial cells.

Conclusion

In brief, *Bacillus* strains play important roles in the costly nitrification–denitrification processes and have some economic advantages (Kim *et al.* 2005).

The present results indicate that the inoculation of probiotic bacilli at concentrations of 0, 10^6 , 10^7 , 10^8 , CFU/L resulted in bioremediation of effluent or waste water of fish cultivation system. The inoculation of 10^8 CFU/L of the bacterium resulted in the highest bioremediated rate, hence 10^8 CFU/L of the *Bacillus* probiotics was selected as the optimal amount of inoculum and the best time of performance of this dose was 72 h.

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اثر غلظت‌های مختلف باسیلوس‌های مفید بر اصلاح زیستی پساب سیستم پرورش ماهی کپور معمولی (*Cyprinus carpio*)

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چکیده:

مطالعه‌ای 4 روزه با هدف بررسی اثر سویه‌های اصلاحگر باکتریایی باسیلوس لیچنی فورمیس، باسیلوس سابتیلیس، باسیلوس پلی-میکسا، باسیلوس لاتروسپروس و باسیلوس سیرکولانس (پروتکسین آکواتک) بر تنظیم برخی پارامترهای کیفی آب اعم از ازت آمونیاکی کل، ازت نیتريت، ازت نیترات و کدورت انجام شد. پساب سیستم پرورشی ماهی کپور معمولی در این آزمایش مورد استفاده قرار گرفت. ترکیبی از باسیلوس‌ها با غلظت 1×10^6 ، 1×10^7 و 1×10^8 واحد کلنی باکتریایی به طور مستقیم به هر لیتر از پساب در مخازن تیمارهای باکتریایی اضافه شد و تاثیر آن‌ها بر پارامترهای مورد نظر در فواصل 24، 48 و 72 ساعت مورد بررسی قرار گرفت. اثر زمان و غلظت در یک طرح کاملاً تصادفی خرد شده در زمان مطالعه شد. نتایج پژوهش حاضر نشان داد که زمان‌ها و غلظت‌های مختلف باکتریایی می‌توانند پارامترهای کیفی آب را تحت تاثیر قرار دهند. زمان و غلظت و اثر متقابل آنها تفاوت معنی‌داری ($P < 0.05$) بر تغییرات ازت آمونیاکی کل، ازت نیتريت، ازت نیترات و کدورت داشت. شمارش مجموع باکتری‌های موجود در آب مخازن اصلاح شده نیز کمتر از مخزن کنترل بود. هدف از این آزمایش، یافتن بهترین غلظت از باکتری و بهترین زمان عملکرد آن‌ها جهت کمک به تعدیل پارامترهای کیفی آب در مخازن پرورش به عنوان محیط زندگی ماهی‌ها، برای افزایش رشد و بازماندگی، اصلاح زیستی پساب، استفاده مجدد از منابع آب و جلوگیری از آلودگی‌های ثانویه بود.

کلمات کلیدی: باسیلوس، درمان باکتریایی، پارامترهای کیفی آب، اصلاح زیستی