ANIMAL HUSBANDRY

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MODERN TECHNIQUES FOR RAINBOW TROUT BREEDING USING BIOTECHNOLOGICAL AND GENETIC APPROACHES FOR WATER DENITRIFICATION

Research article

Abstract

Main environmental pollutants in trout aquaculture are non–ionized ammonium (ammonia) and nitrite. Ammonia is produced by protein catabolism in fish and is excreted from the blood through the gills. Decreased growth, erosion of tissues (kidneys, gills and skin) and degenerative processes in other tisuues and organs, immune suppression and high mortality of fish are associated with the accumulation of large amounts of ammonium in aquatic systems.

An analysis of the literature data showed that the isolated bacterial complex reduces the ammonia content in the trout cultivation system. The treatment group achieved a reduction in mortality and an increase in gains. In addition, a decrease in stress and immune responses was observed when water contains heterotrophic ammonium and nitrite microbial complex (HAN) compared to the control fish group. This review shows that HAN culture applied to water in aquaculture effectively reduced the concentration of non–ionized ammonia. This leads to an improvement in the physiological state of the trout and an increase in its growth. Combined application of strains of two bacterial species *Dyadobacter* sp. and *Janthinobacterium* sp. can be recommended for rainbow trout farming systems as it results in a significant acceleration of *Rainbow trout* growth and maturation.

Keywords: *Rainbow Trout,* bacterial strains, ammonia, aquaculture.

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СОВРЕМЕННЫЕ СПОСОБЫ РАЗВЕДЕНИЯ РАДУЖНОЙ ФОРЕЛИ С ИСПОЛЬЗОВАНИЕМ БИОТЕХНОЛОГИЧЕСКИХ И ГЕНЕТИЧЕСКИХ ПРИЕМОВ ДЕНИТРИФИКАЦИИ ВОДЫ

Научная статья

Аннотация

Основными загрязнителями окружающей среды в аквакультуре форели являются неионизированный аммоний (аммиак) и нитрит. Аммиак вырабатывается при катаболизме белков у рыб и выводится из крови через жабры. Снижение роста, эрозия тканей (почки, жабры и кожа) и дегенерация других тканей и органов, подавление иммунитета и высокая смертность рыб связаны с накоплением большого количества аммония в водных системах.

Анализ литературных данных показал, что выделенный бактериальный комплекс снижает содержание аммиака в системе культивирования форели. В группе лечения было достигнуто снижение смертности и увеличение прироста. Кроме того, наблюдается снижение стрессорных и иммунных реакций у рыб в среде, содержащей гетеротрофные аммонийные и нитритразлагающие бактерии (ГАН) по сравнению с контрольной группой рыб. В настоящем обзоре показано, что культура ГАН, примененная к системе культивирования форели, эффективно снижала концентрацию неионизированного аммиака при выращивании форели. Это приводит к улучшению физиологического состояния форели и усилению ее роста. Комбинированное применение штаммов двух бактериальных видов *Dyadobacter* sp. и *Janthinobacterium* sp. может быть рекомендовано для систем выращивания радужной форели, так как это приводит к значительному ускорению роста и развития радужной форели.

Ключевые слова: радужная форель, штаммы бактерий, аммиак, аквакультура.

1. Introduction

A) Background

Cold-water fish farming is an industrial method of breeding fish, wholly or partly reared in artificial conditions, at a temperature not exceeding +18°C. Aquaculture of cold–water fish farming is carried out in cold water, in contrast to warm-water fish that can only survive for a short time in cold water, and grow after the water warms up above 16–18 °C. Currently, several types of salmons are raised in cold water systems worldwide: *Rainbow trout*, *Brook trout*, *Char*. Trout production is based mainly on by *Rainbow trout* farming [1]. The latter has been introduced and farmed on almost all continents. In the literature, the term "cold–water fish farming" is often replaced by "trout farming". It is well known that during fish growth water is becoming contaminated with increasing amount of ammonia which has a negative impact on fish health and productive characteristics. Natural sources of water also differ significantly in ammonia content. To resolve this matter of the aggregation of hazardous nitrogen containing substances in water, microorganism strains capable to grow at low temperatures of about 15°C and the ability to degrade ammonium and nitrite were selected from environment with subsequent genetic identification and certification [2], [3].

Rainbow trout is a cold–water salmonid fish species that can withstand temperatures in the range of 0–25°C. The optimum temperature for the development of eggs is 6–12°C, for keeping larvae and fry 14–16°C, for adult trout 14–18°C. At a temperature above 20–22°C, the trout stops feeding; when the temperature drops below the optimum, the trout feed consumption also decreases [4]. In winter, trout in fresh water of natural reservoirs withstand temperatures close to zero (freezing). In salt water, trout survives at sub–zero water temperatures of –3–5°C. Trout is an oxyphilic fish, that is, it needs water well saturated with dissolved oxygen, prefers clean and clear waters. The normal vital activity of a trout proceeds at 90–100% saturation of water with oxygen, that is, when the oxygen content is below 7–8 mg/l, the trout dies. The active reaction of the environment (pH) should be close to neutral and not go beyond 6.5–8.5 values. Trout does not like bright sunlight, if possible, hides in the shade, under stones, snags, or goes into deep places. Under natural conditions, it is most active on cloudy days, in the evening and in the early morning. At the same time, unlike other fish, the trout constantly stays closer to the surface of the water, since the filling of the swim bladder with air is carried out only by capturing it from the atmosphere. Therefore, in closed cages, completely immersed in water, as well as in winter in completely freezing reservoirs, fish cannot live. Adult *Rainbow trout* is able to endure oceanic salinity of up to 32%, larvae withstand salinity of 5–8%, fry can live in the range 12–18%, and yearlings prefer 20–25% [5].

Trout females become sexually matured at the age of three or four years and this age vary depending on the location of the reservoir, the climatic conditions of the area and the themperature conditions of the water system. *Rainbow trout* males get matured state one year earlier than females. Spawning begins from March till May in the upper parts of cold–water streams and rivers, in shallow areas with a fast water current on stony and pebbly ground. The female spawns from 500 to 2500 yellowish–orange eggs. Caviar is large, having diameter in the range of 4.0–6.5 mm. The process of caviar development lasts 1.5–2.0 months [5].

In terms of feed behavior adult trout is a predator. In its diet there are small fish, frogs, small or young birds, gammarus, mollusks, larvae, insects, and even rodents. Sometimes it eats its own juveniles. She also uses in her diet. Trout reaches its optimum growth rate at a water temperature of 16–18 °C. For a growing period of 280–300 days, the individual body weight of a trout under these conditions reaches 250–300 g. In closed-cycle plants, on ground and underground waters, where the temperature is constant and close to optimal throughout the year, trout are grown with the expected growth rate. When rearing trout in systems with natural water temperature, such as open systems, the growth rate depends on the climatic conditions, especially on the water temperature and the nature and size of reservoirs [1], [4].

The mechanical filter can not absorb all organic substances; the smallest particles can go through it in the same way as dissolved chemicals like phosphate or nitrogen. Phosphate stuff is biologically neutral with no toxic impacts on trout, but nitrogen contained in free ammonia (NH3) is toxic and must be transformed by bacteria immobilized on a biofilter to harmless nitrate forms [6], [7], [8]. The degradation of organic substances and ammonia can be achieved by metabolism carried out by certain strains and species of bacteria fixed to a biofilter. Heterotrophic bacteria, utilizing dissolved oxygen, oxidize organic substances converting them into ammonia, carbon dioxide, and sludge. Nitrogen converting bacteria make during metabolic processes ammonia to nitrite. Thre next step is a conversion nitrite to nitrate [9]. The efficiency of this process of bioconversion depends largely on the water temperature, the pH value in the environment. There are different kinds of biofilters with different types of plastic matrices. The plastic biofilter with immobilized bacteria is resistant to chemicals, temperature, UV and biodegradation. For the operation of the biofilter, pumps with air diffusers are required. Particularly valuable are biofilters with heterotrophic ammonium and nitrite microbial complex (HAN). Biofilters are particularly suitable in recirculating aquaculture systems (RAS) [10].

B) Bacteria selection

Bacterial strains isolated from environment with high potential to utilise ammonium in water (efficiency of up to17.12–24.75% removal of NH4–N), samples no. 2 (EO), 6 (IO), 62 (P), 68 (EO) and 117 (P) had excellent growth characteristics. Bacterial strain numbered 68 (no. 68) was selected as one of the the best candidate strains in terms of propagation and ammonium degrading capacity. What is unexpected is that the strain no. 68 was discovered and isolated from a water source with quite low ammonium content [11]. Since the new microorganism strains in aquaculture system is potentially dangerous, these strains should be well characterized in terms of non-pathogenic properties. This is made by tests and prior to use selected strains must be geneticallycharacterised to avoid unintentional or intentional contamination with undesirable bacteria [12]. Nowadays the “gold standard” genetic technique for bacteria certification is16S rRNA sequencing which was applied for genetic identification of isolated heterotrophic ammonium-degrading bacteria [9].

Using this genetic approach many bacterial species and strains were distributed into clusters showing their genetic relatedness. It was demonstated that sequence of16S rRNA genes of *Dyadobacter hamtensis* (NR 042226.1) proved to be genetically (94.62% nucleotide similarity) to the highly productive strain No. 68 which was isolated earlier. Microscopic analysis of the strain no. 68 showed that bacterial cells tend to be aggregated into chains like bacteria of streptomyces group. This property may be useful because chains easily form links with biofilter surface, thus facilitate their immobilisation. The selected strain of *Dyadobacter* sp. no. 68 is neutral and has no pathogenic activity, the bacterium grows fast, ammonium degrading activity is high. So, combination of thses biological trait made it the best candidate for further use in aquaculture. All isolated strains, when placed in the nitrogen containing medium, showed distinct ability to remove nitrite, and their degradation activity varied from 10.57% to 49.37% of nitrite in water after 10 days of incubation. Among other selected environmental strains, no. 100 also demonstrated high nitrite degraded efficiency [11].

A group of the best heterotrophic nitrite-degrading bacteria included strains no. 3 (P), 9 (IO), 16 (P), 84 (IO), 100 (P), 154 (EO), and all these strains were characterized by sequencing of 16S rRNA genes. The calculation of genetic distances between individual strains showed that nitrite–removing strains, no. 84, no. 100 and no. 154 were genetically close to genus *Janthinobacterium.*This is direct evidence that selected strains having nitrite removal activity all belong to the mentioned above bacterial genus. Furthemore, *Janthinobacterium svalbardensis* (KR 085903.1) known as non-pathogenic bacteria is genetically close with 95.33% sequence similarity to strain no. 100. All these data indicate on usefulness of selected bacterial strains as efficient renovers of nitrogen–containing substances.

Therefore, these strains were chosen for further analysis as the optimal nitrite degrading microorganisms. Earlier it was demonstated that inherent feature of bacyteria belonging to genus *Janthinobacterium sp.* and *Dyadobacter* sp. exhibit pronounced nitrification potential resulting in significant nitrite removal in water systems [6], [13]. Thus, bacterial activity converts nitrite to nitric oxide by special enzyme called nitrite reductase. On the other hand, there are some reports indicating on pathogenic potential in aquaculture of certain *Janthinobacterium* species [14].

2. Materials and methods

Adaptation of selected species of bacteria to low temperature environment.

Naturally, Dyadobacter sp. and Janthinobacterium sp. bacteria are not adapted for efficient propagation and conducting enzymatic processes at low temperatures around 15°C. Rainbow trout prefers cold water with optimal temperature for growth around 14–18°C. Therefore, for efficient use of biofilters with immobilized selected strains of bacteria the latter should be get used to develop at low temperature in order to be effectively introduced into trout aquaculture [13].

Experiments aimed at the adaptation of bacteria to low temperature were designed to grow selected strains at contrast conditions of low (15°C) and ambient temperatures (22.3 ± 2.8°C). Under both conditions ammonium depletion was observed. The data obtained showed that Dyadobacter sp. was able to demonstrate sufficient ammonium degrading activity at low temperature [11]. Process of ammonium degradation was initially observed since the 6th day of observation and detected till the 15th day. Dyadobacter sp. in laboratory tests demonstrated the most pronounced ammonium removal activity that is why this bacterium was selected for further tests in aquaculture environment. In contrast to Dyadobacter sp., Janthinobacterium sp., demonstrated its activity earlier starting on day 3 both at low temperature and ambient temperature. Nitrite degradation activity at low temperature was lower than that at ambient temperature during the experimental period of day 3 to day 9. During days 12 to 15, the highest nitrite degrading activity of 11.6 and 13.25 mg/l per day was observed at 15°C and ambient temperature, respectively. Thus, Janthinobacterium sp. has a capacity to remove nitrite at ambient temperature of water around 15°C. This bacterium along with Dyadobacter sp. can be used in immobilized form on filters in recirculated water systems (RAS) [11].

3. Results and discussion

A) Results of simultaneous application of *Dyadobacter* sp. and *Janthinobacterium* sp. for denitrification of water

No wonder, that the concentration of ammonia in control group (no immobilized bacteria on a filter) in closed water system used in experiment exceeded significantly biologically justified and practicallyrecommended values for *Rainbow trout* farming. The concentration of non–ionized NH3 in the heterotrophic ammonium and nitrite microbial complex (HAN) group (8.8 ± 1.8 μg/l) was substantially less than that of control group (13.2 ± 2.1 μg/l) (Table 1) [11]. This proves that *Dyadobacter* sp. was efficient as a remover of non–ionized ammonia in closed water system. The bacterium ensures normalization of this stuff content to a level below the recommended limit. In parallel, application of HAN culture also gives rise to a significant decrease in the nitrites and nitrates concentration in the experimental group compared with the control. In the HAN experimental group, the ammonium concentration is expected lowered by transforming this substance into nitrite by *Dyadobacter* sp. bacterial strain. Result is that nitrite accumulation occurs, the process which is also harmful for *Rainbow trout.* This problem is elevated by the simultaneous activity of *Janthinobacterium* sp., which converts nitrite to nitrate. Nitrite concentration of 62 ± 11 μg/l–1 in the HAN experimental group higher than that of control group but still significantly lower suggested values of 1000 μg/l^{-1}. The table contains data concerning mean values ± SD for three repeats of experiment. The water pH parameter in control group was lower compared to the experimental group with HAN complex. It is known that hydroxamic acids are generated by activity of heterotrophic bacteria leading to nitrification of products [11]. So, ammonium and nitrite concentrations in water environment *Rainbow trout* aquaculture are reduced due to the metabolic activity of *Dyadobacter* sp. and *Janthinobacterium* sp. In experiments conducted for 2 weeks strains of *Dyadobacter* sp. and *Janthinobacterium* sp. (cold-adapted strains no. 68 and no. 100, correspondingly) demonstrated sufficient ammonium and nitrite degrading activity. Microbial species of *Dyadobacter* sp. (strain no. 68) and *Janthinobacterium* sp. (strain no. 100) were used in combined manner (mixed culture with ratio 2.5:1, correspondingly) in water for *Rainbow trout* farming. In 9 days of experiment, the degrading efficiency of this combination of bacteria for non–ionized ammonia and nitrites was examined [11].

Thus, the change in pH in the experimental group may be associated with nitrification. Other water parameters indicating water quality include dissolved oxygen, and water temperature, statistically did not change with activity of HAN group compared to the control. *Rainbow trout* experiences less stress when incubated under conditions with HAN bacterial complex. This may explain higher productivity of fish cultures in water systems supplied with HAN complex.

Table 1 – The effect of two–species microbial complex on the content of non-ionized ammonia, nitrite, nitrate, and other parameters in the breeding of *Rainbow trout* (n = 50) during 9 days of observation [11]

Parameter values	Groups		
	HAN	Control	Recommended values [15]
NH_3–N, mkg/l^{-1}	8,8 ± 1.8[a]	13,2 ± 2,1[b]	12,5
NO_2–N, mkg/l^{-1}	62 ± 11[a]	31 ± 15[b]	1000
NO_3–N, mkg/l^{-1}	15,6 ± 5,4	13,1 ± 4,1	<400
Temperature, °C	14,2 ± 1,2	14,1 ± 1,2	<16
pH	6,9 ± 0,1[a]	6,3 ± 0,2[b]	6,5 – 8,5
Dissolved O_2, mg/l^{-1}	8,7 ± 2,1	8,5 ± 3,2	>6

Note: values within the same line with superscripts "a" and "b" have statistically significant difference (p <0.05)

B) Results of simultaneous application of *Dyadobacter* sp. and *Janthinobacterium* sp. for fish traits

Change in environmental conditions in water can result in propagation of opportunistic and even pathogenic microorganisms. This can also have harmful effect on fish ability to consume feed stuff. In its turn, this results in lower rate of fish growing and survival abilities. The experiments with HAN complex demonstrated that the latter is effective tool to significantly raise survival rate in *Rainbow trout*. The data obtained prove that not only survival rate is higher in HAN group but also final weight has a tendency to be higher in HAN group compared to control. The experiment was done in three repetitions (Table 2).

Table 2 – Weight and survival rate of *Rainbow trout* in HAN experimental group and control group during 9 days of observation [11]

Fish groups	Phenotipic and survival traits		
	Intial weight, g	Final weight, g	Survival rate, %
Control	50,7 ± 3,9	57,8 ± 3,4	72,3 ± 6,2[a]
HAN	50,7 ± 3,9	60,8 ± 4,6	86,4 ± 7,3[b]

Note: values within the same column with superscripts "a" and "b" have statistically significant difference (p <0.05)

C) Revealed parameters concerning hematology in *Rainbow trout* as an impact of HAN complex supplement

In fish breathing organ is called gills which is extracting oxygen from water and transmit it to a blood stream for subsequent oxygen supply to all other organs. Any anomaly in the respiratory system of fish theoretically can negatively affect many hematological parameters. Thus, some hematological biochemical and morphological characteristics in fish breeding in water with HAN complex were compared with corresponding characteristics in fish of the control group. Analysis of blood characteristics including concentration of hemoglobin, hematocrit value, red blood cell (RBC), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV), did not differ between two groups under comparison (Table 3). So, no significant hematological changes which might have negative effect in fish while applying HAN complex were detected [11].

Table 3 – Manifestation of hematology parameters (hemoglobin content, hematocrit value, RBC, MCV, and MCHC) in groups of *Rainbow trout* after 9 days of experiment [11]

Group	Indicators				
	Hemoglobin, g/dl	Hematocrit, %	RBC, 10^6 μl^{-1}	MCV, fl	MCHC, %
Control	63,8 ± 2,2	40,0 ± 1,3	1,2 ± 0,1	318,7 ± 12,6	51,41± 1,5
HAN	60,0 ± 1,5	38,5 ± 1,8	1,3 ± 0,1	304,0 ± 8,2	47,6 ± 1,2

Thus, a tendency to the hemoglobin and hematocrit slight increase in control group may be due to the need to increase the oxygen–carrying capacity to ensure sufficient oxygen in fish tissues. However, further studies over longer experimental time, higher fish load in water system, longer HAN exposition time, will be reasonable to better understand theoretical and practical potential of microbial complexes for fish breeding especially in recirculating water systems. This would allow getting statistical significance when comparing certain parameters in experimental and control groups. Environmental origins of fish stress can be discriminated into acute (short time, severe factor) and chronic (longer time, less severe factor). It is known that cortisol level rises under chronic stress, when the body needs more energy otained from glucose. This process is associated with pronounced suppression of fish innate immune system. Ammonium and nitrite are among strong stress factors in *Rainbow trout* [16], [17].

Adding HAN complex promotes decreas in cortisol along with glucose levels in fish when take into account control values. This is indirect evidence that fish experiences less environmental stress when bred in water system with equipped with

biofilters with HAN bacterial complex [18]. The propagation of opportunistic microorganisms is also accelerated under fish conditions of chemical–induced stress. This usually triggers an acute response of fish immune system. The results of many investigations demonstrate that such parameters as total protein in blood serum, proteins of globulin fraction, complement activity, and activity of lysozyme in the experiment did not reach values in the control group by the end of the experiment [16]. These observations also support an idea that HAN complex facilitates comfort environmental conditions for fish and thus reducing stress [11].

D) Genetic data of *Rainbow trout* raising

Rainbow trout is a genetically well–characterised fish species. In particular, the tetrapolidity of trout has been published [1]; microsatellite DNA has been used worldwide to reveal the genetic structure and phylogeny of salmonid fish species [19]. A transgenic salmon has been on the market for a number of years demonstrating a high productive performance of such fish. A hormone gene from Chinook salmon was inserted into salmonid genomic DNA. The gene expression was facilitated by a promoter linked to a gene construct from the eelpout antifreeze protein gene. The fish growth in transgenic salmon exceeded that of non–transgenic fish by a factor of 2 as a recult of elevated hormone concentration in blood and a whole–year corresponding gene expression.

Another application of genetic approaches is generation of higher rate of females using phenomenon of the sex reversion, thus, unisex individuals are obtained. Reversed males mature later and therefore grow faster before maturation; more efficiently assimilate feed and produce caviar, an expensive food product. Sex is determined genetically and hormonally, more often genetically. Females carry the XX sex chromosomes (all gametes carry an X chromosome), males carry the XY sex chromosomes (X and Y gametes). When gametes X and X merge, a female is obtained, gametes X and Y produce a male. The presence of the X chromosome leads to the synthesis of estrogen – the female sex hormone, the development of the body follows the female path. The presence of the Y chromosome leads to the synthesis of androgens – male sex hormones, the development of the body follows the male path. Both hormones are present in the fish organism, but one of them prevails, determining the development of the organism and, first of all, the gonads. Therefore, if a larva with chromosomes XX is treated with androgens, its development will follow the male path, and the gonads will produce spermatozoa containing only the X chromosome. This procedure is called gender reversal. The androgen, methyltestosterone, is used for sex reversal. Liquid methyltestosterone is added to the feed in an amount of 1–3 mg/kg (tablets can be diluted in ethyl alcohol). The feed is treated with an alcohol solution of methyltestosterone using a sprayer and air–dried in a dark place. The larvae are fed of processed food according to the established protocols. Feeding with processed feed begins when the larvae switch to active feeding. Regular feeds are not used during the sex reversal process [19]. After the reversion process is completed, the juveniles are fed a normal fish diet.

Over the years, researchers have paid considerable attention to DNA technologies in breeding, in particular, the use of allelic variants of individual genes in animals associated with productive traits [20], identification of bacterial strains [21] and genome–wide analysis of polymorphic sites in fish [22]. Another interesting fish breeding approach was the use of triploids. Scientists have long noticed that in salmonids there are triploid and tetrapolid individuals, ploidy mosaics, and there are also duplicated individual genes in the genome [23]. Experimentally, tripolides can be obtained by intraperitoneal transplantation of immature germ cells of spermatogonia into embryos, followed by their growth to an adult state. Eggs and sperm are taken from the obtained triploid individuals, fertilization and fry rearing are carried out. The offspring consists of normal diploids, triploids and tetraplolids. In some cases, by analogy with tetraplolid plants, the fish showed faster growth in comparison with ordinary diploid individuals.

4. Conclusion

Analysis of literature demonstates efficiency of application of biofilters with selected strains of bacteria in recirculating aquaculture systems (RAS). Certain strains of bacteria like *Dyadobacter* sp. *and Janthinobacterium* sp. provide significant water cleaning along with degradation of nitrogen–containing harmful substances. This ensures better growth and health of *Rainbow trout* in aquaculture. Further studies aimed at the generation other more efficient bacterial strains are needed in order to get higher ecological standards in fish breeding farms.

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Conflict of Interest

None declared.

Конфликт интересов

Не указан.

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