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水产养殖中好氧反硝化细菌的筛选及评价研究进展

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摘　要: 高密度、集约化水产养殖中氨氮、亚硝酸盐等有害氮源大量积累,严重破坏水环境并损坏水产动物的健康,对水产养殖行业造成极大危害。好氧反硝化细菌可将有害无机氮还原为氮气溢出,有效改善水质,而菌种筛选是好氧反硝化细菌研究和应用中一个关键环节。本文对好氧反硝化细菌菌种的筛选、评价方法进行归纳、总结,从样品采集、培养基配制、培养方式及评价方法等方面探讨适合水产养殖的方法和标准,以期对水产中好氧反硝化菌种筛选和评价提供参考,并对未来的研究方向进行了展望。

关键词: 水产养殖;好氧反硝化细菌;筛选方法

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我国是全球最大的水产养殖国家,养殖水产品总量逐年增长。据统计,2019 年我国养殖水产品总产量达 6 450 万 t,超过世界养殖水产品总量的 70%,为优质蛋白质的供给以及国家的粮食安全做出了巨大贡献。目前,高密度、集约化已成为我国水产养殖的主要模式[1],然而,养殖密度的不断提高极易打破池塘原有的生态平衡,过多的残饵、粪便无法被池塘中的微生物分解利用,导致氨氮、亚硝酸盐等有害物质积累,影响养殖动物健康[2]。此外,不经处理的养殖废水排放到外环境中产生面源污染,危害养殖业的可持续发展。生物脱氮不仅安全环保,而且具有可续持性,近年来新发现的好氧反硝化细菌因其能够在有氧的条件下进行反硝化作用,脱氮彻底,而且具有适应性强、生长速度快及容易控制等潜在优点[3-5],逐渐成为脱氮益生菌中研究和应用的热点。

在益生菌研究和应用中,菌种筛选是一个十分重要的环节。然而,由于在菌种筛选过程中候选菌株众多而且筛选条件各异,水产养殖常用的益生菌菌种筛选的方法、标准非常繁杂,导致菌种筛选效率低下;其次,实验室筛选得到的菌种在养殖试验验证过程中,其功能往往不能较好的重现,这主要是由于实验室筛选益生菌的条件或许同其应用的环境有所区别,从而使益生菌应用后在靶位点难以生长、定植及发挥功能[6];此外,某些对哺乳动物有益的微生物应用于水生动物可能会产生不利影响。因此,在益生菌菌种筛选过程中制定适合水产养殖的方法、标准就显得非常重要。目前,已有很多关于水产养殖中好氧反硝化菌种筛选和应用的研究[3,7-10],但尚未见到对该菌种筛选及评价方法进行系统综述的报道。因此,本文对水产养殖中好氧反硝化细菌的筛选、评价方法进行归纳、总结,以期对好氧反硝化细菌筛选和评价标准提供参考,并对未来的研究方向进行了展望。

1　菌种筛选流程

随着研究的深入,已有大量好氧反硝化细菌的研究报道,主要菌种包括:假单胞菌(*Pseudomonas* sp.)[8]、芽孢杆菌(*Bacillus* sp.)[11]、副球菌

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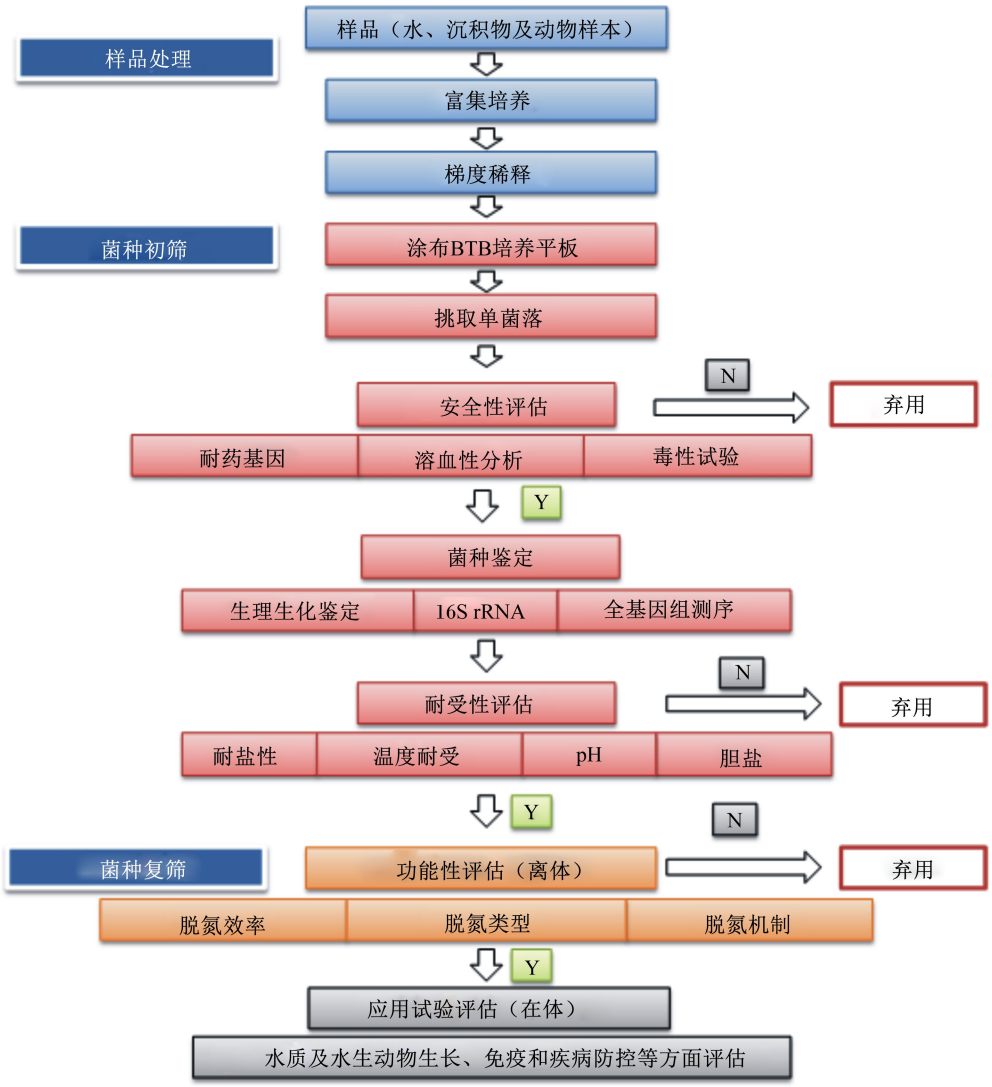
(*Paracoccus* sp.)[3]、海杆菌(*Marinobacter* sp.)[12]、盐单胞菌(*Halomonas* sp.)[13]和红球菌(*Rhodococcus* sp.)[14]等,然而,尚缺乏对该菌种筛选和评价的系统报道。通过归纳、总结国内外相关文献,好氧反硝化菌种筛选流程和标准如图 1 所示。具体而言,好氧反硝化细菌的筛选包括了样品采集、富集培养、安全性评估、菌种鉴定、耐受性评估、功能性评估及应用试验评估等主要组成步骤。通过层层筛选,获得的菌种需要符合安全性、适应性、功能性和便利性等特点[15]。



BTB:溴百里酚蓝 bromothymol blue;Y:通过 pass;N:未通过 fail。

图 1　好氧反硝化菌种筛选流程和标准

Fig.1　Screening procedure and criteria of aerobic denitrification bacteria

2　样品处理

理想的益生菌,应该能够在宿主的肠道或环境中定殖、建立和繁殖,然而,某些应用于水产动物的益生菌常出现相对无效的情况,这或许是由于非鱼类来源的益生菌无法在其肠道中生存或保持最佳的活菌数[16-17]。基于此,好氧反硝化菌种分离常采集的样本最好选取池塘的水样、沉积物、微生物絮团及水产动物肠道等样本,从这些样本中筛得的菌种具有更好的适应性,即原位/宿主益生菌策略(autochthonous probiotics/host-associated probiotic)。以往研究也发现,在水、泥、沉积物及

生物絮团样品中更易筛得好氧反硝化细菌,如滨海芽孢杆菌(*Bacillus litoralis*)[18]、枯草芽孢杆菌(*Bacillus subtilis*)[19]、施氏假单胞菌(*Pseudomonas stutzeri*)[20]及 *Paracoccus saliphilus*[21]等菌种,且具有较好的适应性。此外,采集的样本还可以用高浓度的硝酸盐、亚硝酸盐及铵盐作为唯一氮源进行富集培养[22],培养过程中需要进行间歇曝气以达到好氧反硝化微生物数量增加,同时确保目的菌种的好氧反硝化性能不退化或可得到进一步加强[23-24],通过富集培养可大幅度提高后续菌种的筛选效率。

3 菌种初筛

好氧反硝化菌株初步筛选可应用溴百里酚蓝(bromothymol blue,BTB)固体培养基[25],接种的细菌由于反硝化作用使 pH 升高,从而产生蓝色晕圈,选取蓝色菌落即可实现菌种的初筛。应用该方法,Chen 等[5]从循环水养殖系统的生物滤池中分离到好氧条件下能脱氮的菌株 Z1 和 Z8,Song 等[11]分离筛选得到高效脱氮的好氧反硝化菌株凝结芽孢杆菌(*Bacillus coagulans*)XY-6,Shao 等[26]也筛选得到脱氮性能较好的好氧反硝化菌种 *Pseudomonas* sp. B5。根据筛选流程,初筛获得的菌种还需要进行安全性评估、菌种鉴定及耐受性评价。

3.1 安全性评估

众所周知,水产养殖中应用的微生物菌种必须是安全的,对鱼虾不能有致病性。然而,Fu 等[27]从 92 种动物用益生菌产品中分离出 123 个益生菌菌株,其中 45 个菌株对抗生素耐药,33.7% 的益生菌产品被肺炎克雷伯菌等危及生命的病原体污染,而且还发现了炭疽毒素阳性的蜡样芽孢杆菌(*Bacillus cereus*)菌株,这对水产动物及人类的健康构成巨大威胁。可见,筛选用于水产养殖的益生菌菌种,进行安全性评估为最重要一环。好氧反硝化候选菌种不能含有任何质粒编码的耐药基因或基因簇[28],不产生溶血圈,不含有致病基因,不生成毒性代谢产物,且需要结合体内的急性、慢性毒性试验进行验证[29]。

3.2 菌种鉴定

好氧反硝化菌种鉴定方法同常规微生物菌种鉴定方法类似,包括常规形态学观察、生理生化分析、16S rRNA 测序和全基因测序等。经过菌种鉴定,可获知菌株的种、属等信息,这对进一步研究菌种的性能及实际应用提供了重要参考。具体而言,以饲料添加剂形式饲喂的菌种需符合各个国家规定的菌种目录,如美国食品与药物管理局(FDA)和美国饲料控制官员协会(AAFCO)公布的可直接饲喂微生物菌种名单(46 种)、欧盟准许饲喂的菌种目录(72 种)、我国《饲料添加剂品种目录》允许添加的微生物菌种目录(35 种);而改良水体环境的菌种目前尚未有明确规定,但需要符合之前所提到的安全性要求。

3.3 耐受性评估

益生菌能够发挥效果的关键因素之一为耐受所应用的环境,并且能够在效应位点定植生长,维持较高的活菌水平,而后才能发挥功效(图 2)。不同于陆生动物,益生菌作用水产动物的效应位点除了动物肠道外,还有水环境。因此,筛选的候选好氧反硝化菌株需要耐受所应用的外环境,包括温度、盐度和 pH 等,饲喂的益生菌还需经过胃酸、胆盐等的耐受性评价[30]。一般而言,能够较好满足这一要求的就是选用池塘或水产动物肠道的土著菌群,Boutin 等[31]在研究美洲红点鲑(*Salvelinus fontinalis*)时发现,同外源益生菌相比,本土的益生菌不会干扰鱼皮肤黏液菌群,是调节鱼类微生物群落更好的选择;Ahmmed 等[32]也发现,源于对虾肠道的乳酸杆菌能够更好地适应动物肠道,而且可抑制病原弧菌的生长繁殖;Muthukrishnan 等[33]研究发现,本土菌株越南芽胞杆菌(*Bacillus vietnamensis*)VCM5、*Bacillus vietnamensis* VCM8 和支气管戈登菌(*Gordonia bronchialis*)VCM12 可显著降低对虾养殖废水中亚硝酸盐含量,显示出较好的适应性。此外,某些需要在饲料制作过程中添加的反硝化菌制剂,还需考虑工厂化水产饲料生产的高温、高压耐受性,在这种情况下,应用芽孢孢子可能更为适宜。

4 菌种的复筛

好氧反硝化细菌的复筛包括:应用选择性培养基(selective culture medium)计算菌种脱氮效率、判断脱氮类型以及借助分子生物学和组学手段研究脱氮机制等步骤[34-37]。

4.1 脱氮效率研究

在好氧反硝化细菌脱氮效率的研究中选择性培养基的配方同养殖水体的化学组成存在差异,

如表 1 所示,选择性培养基常用氮源为 NH_4Cl、$NaNO_3$ 及 $NaNO_2$ 等,常用的碳源为琥珀酸钠、柠檬酸钠、葡萄糖、蔗糖和甘油等。然而,在水产养殖水体中常为有机碳源缺乏,而且氮源变为营养丰富的有机氮源[18]。筛选培养基的富碳特性在实际的养殖环境中很少存在,池塘养殖环境多为低碳高氮,不同于工业污水可以额外提供大量廉价有机碳源,养殖中大量有机碳源的应用可能会造成水产动物缺氧风险及病原微生物滋生等问题出现。基于此,Ma 等[38]直接配制 0.05 g/L 的 $NaNO_2$ 溶液,并在其中仅补充 1 g/L 粉碎的鱼饲料及粪便以模拟池塘中积累的有机物质,以此反硝化细菌池塘模拟培养基(pond-simulating denitrification screening medium,PSDSM)进行菌种筛选,获得了脱氮效率理想的好氧反硝化菌株 *Bacillus subtilis* M 7-1,同时,大田应用试验证实,该菌株对养殖池塘中的无机氮也具有较高的降解率[39]。Cui 等[40]在有机氮(蛋白胨、尿素)存在的情况下,研究反硝化细菌海洋着色菌(*Marichromatium gracile*)YL28 脱氮性能,发现存在有机氮源且添加海藻寡糖后,YL28 对无机氮的去除能力显著增强。可见,在反硝化细菌的筛选过程中,培养基中氮源、碳源的选择及适宜的碳氮比关系着筛得菌株的脱氮性能,研究发现,大多数好氧反硝化细菌的最佳碳氮比在 8~10[41-42],然而,养殖池塘中一般碳氮比在6~8,因此在筛选培养基配制需考虑这一差异,或在应用中补充缓释碳源以确保菌种的脱氮功能[43]。

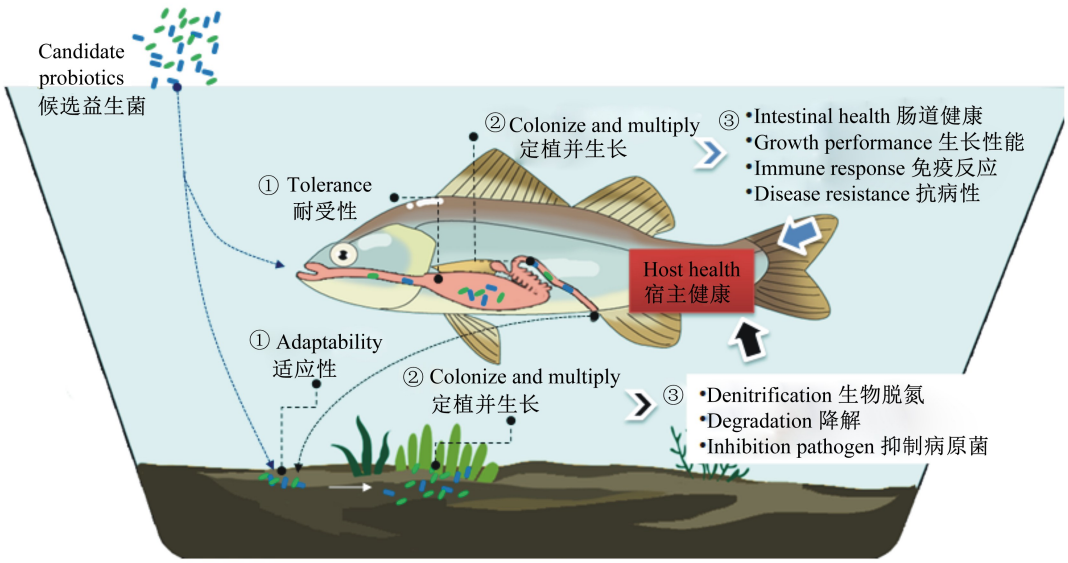


图 2　益生菌在鱼类肠道和水体中定植后的作用

Fig.2　Role of probiotics colonization in fish gut and water

温度是反硝化过程的重要参数之一,已有研究显示,好氧反硝化细菌脱氮的适宜温度范围为 25~37 ℃[37,44-45]。因此,在菌种筛选的过程中培养温度可设定为 28~30 ℃(表 1),但考虑冷水鱼养殖及春秋季节水体脱氮的需要,嗜冷(10~15 ℃)好氧反硝化细菌的筛选也尤为重要。He 等[46]研究发现,好氧反硝化菌台湾假单胞菌(*Pseudomonas taiwanensis*)在 15 ℃下对硝酸盐的去除率为 100%,具备低温脱氮能力。然而,随着温度的降低,菌种的反硝化效率也会随之降低;Saleh-Lakha 等[47]研究表明,在 10 ℃条件下,好氧反硝化菌孟氏假单胞菌(*Pseudomonas mandelii*)同硝化和反硝化作用相关的基因表达发生滞后和延迟。由此可见,培养温度的设定需要综合考虑菌种最适条件及所应用的环境这 2 方面因素。

盐度也影响着好氧反硝化细菌的脱氮效率。研究表明,海水养殖的盐浓度(20‰~35‰)已可抑制常见细菌的酶系统并可发生溶菌作用[48]。在反硝化方面,Deng 等[49]研究表明,在高盐浓度下,反硝化过程的功能基因 *nirK* 和 *nosZ* 的表达丰度

大大降低,抑制了细菌的反硝化活性。而同时,耐盐好氧反硝化细菌也已有研究报道,Al-Rubaye等[50]已从盐芽孢杆菌属(*Halobacillus*)、海源菌属(*Idiomarina*)、大洋芽胞杆菌属(*Oceanobacillus*)和枝芽孢菌属(*Virgibacillus*)等种属中分筛到反硝化嗜盐菌;Li等[51]从青岛胶州湾海水沉积物中分离出1株反硝化海洋嗜盐菌弧菌;而Mével等[52]分离筛选的*Bacillus* sp.能够在16 g/L的NaCl中进行好氧反硝化作用,这些研究对筛选海水养殖中应用的耐盐好氧反硝化细菌提供依据。

通过调整摇床转速以提供好氧反硝化细菌生长所需的溶氧,一般而言摇床转速设置为150~200 r/min(表1)。然而,不同菌种适宜的溶氧浓度不同,即便相同种类的好氧反硝化细菌在不同溶氧情况下脱氮能力也存在差异[4],比如某些好氧反硝化细菌对高溶氧具有较高的耐受。Robertson等[53]研究发现,在溶解氧浓度为80%~90%的培养基中,泛硫代酵母也具有反硝化酶活性;无色杆菌属(*Achromobacter*)、不动杆菌属(*Acinetobacter*)和假单胞菌属(*Pseudomonas*)能够在溶氧浓度为3~10 mg/L条件下进行好氧反硝化脱氮[54],而芽孢杆菌则可在溶氧浓度为3.93~7.65 mg/L条件下发生反硝化作用[55]。但考虑到微生物同养殖动物可能存在的争氧问题,筛选兼具有好氧和厌氧反硝化能力的微生物或更具有实用性[56]。

4.2 脱氮类型研究

在脱氮类型方面,能够同时进行异养硝化-好氧反硝化的菌株具备更高的脱氮效率,如*Paracoccus saliphilus* SPUM、*Bacillus litoralis* N31、*Marinobacter* sp.、*Bacillus cereus* PB45及盐田盐单胞菌(*Halomonas campisalis*)等(表1)。然而,不同菌种脱氮效率、脱氮类型有较大差异,这或许同反硝化细菌所含酶系的种类和数量不同有关。廖绍安等[57]应用间歇曝气法分筛获得嗜麦芽寡养单胞菌(*Stenotrophomonas maltophilia*),测序发现该菌株未发现亚硝酸还原酶基因*nirK*序列,只鉴定了*nirS*基因序列;从废水处理的活性污泥中分离筛选的盐单胞菌属细菌(*Halomonas* sp.)则鉴定出多种脱氮相关的酶类,可同步发生硝化和反硝化反应,脱氮效率较为理想[58];Wan等[59]对脱氮类型为好氧反硝化的假单胞菌yy7进行分析,确定该菌株含有*nirK*、*norB*和*nosZ*等多个同反硝化相关的基因,这些功能基因同菌种的反硝化性能密切相关。

4.3 脱氮机制研究

筛选的好氧反硝化菌株可通过PCR及全基因组测序获取脱氮相关的酶系基因,从而进一步分析其脱氮机制。如表2所示,反硝化反应在硝酸还原酶(nitrate reductase)、亚硝酸还原酶(nitrite reductase)、一氧化氮还原酶(nitric oxide reductase)及一氧化二氮还原酶(nitrous oxide reductase)的催化作用下进行。

表1 好氧反硝化细菌筛选培养基及筛选条件

Table 1 Screening medium and screening condition of aerobic denitrification bacteria

样品(来源) Samples (source)	选择性培养基(每升含) Selective medium (per liter contained)	筛选条件 Screening conditions	菌株 Strains	脱氮类型 Denitrification mode	参考文献 Reference
水样(虾池) Water (shrimp pond)	$(NH_4)_2SO_4$ 3.035 g,NaCl 0.585 g,KH_2PO_4 0.1 g,KCl 0.075 g,$CaCl_2 \cdot 2H_2O$ 0.147 g,$MgSO_4 \cdot 7H_2O$ 0.049 g,HEPES缓冲液47.66 mL,TE溶液5 mL	28 ℃、180 r/min	*Paracoccus saliphilus* SPUM	异养硝化-好氧反硝化	[21]
水样(虾池) Water (shrimp pond)	琥珀酸钠6.5 g,$(NH_4)_2SO_4$ 0.25 g,$K_2HPO_4 \cdot 3H_2O$ 1.5 g,KH_2PO_4 0.45 g,$MgSO_4 \cdot 7H_2O$ 0.05 g,$FeSO_4 \cdot 7H_2O$ 0.01 g,$MnSO_4 \cdot 4H_2O$ 0.01 g,NaCl 30 g	28 ℃、160 r/min	滨海芽孢杆菌(*Bacillus litoralis*)N31	异养硝化-好氧反硝化	[18]
水样(鲈鱼池) Water (largemouth bass pond)	柠檬酸钠1.31 g,KNO_3 0.181 g,NH_4Cl 0.096 g,KH_2PO_4 1 g,K_2HPO_4 5 g,$MgSO_4 \cdot 7H_2O$ 0.2 g,TE溶液1 mL	30 ℃、180 r/min	假单胞菌(*Pseudomonas* sp.)	好氧反硝化	[9]

续表 1

样品(来源) Samples (source)	选择性培养基(每升含) Selective medium (per liter contained)	筛选条件 Screening conditions	菌株 Strains	脱氮类型 Denitrification mode	参考文献 Reference
水样(草鱼池) Water (grass carp pond)	柠檬酸钠 5.66 g,Na_2HPO_4 7.9 g,$NaNO_3$ 0.841 5 g (DM)/$NaNO_2$ 0.683 g (NDM)/NH_4Cl 0.529 6 g(NM),KH_2PO_4 1.5 g,$MgSO_4 \cdot 7H_2O$ 0.01 g,TE 溶液 2 mL	30 ℃、200 r/min	施氏假单胞菌(*Pseudomonas stutzeri*) F11	反硝化	[20]
		30 ℃、200 r/min	枯草芽孢杆菌(*Bacillus subtilis*) SC02	反硝化	[19]
		30 ℃、180 r/min	地衣芽孢杆菌(*Bacillus licheniformis*) BSK-4	好氧反硝化	[60]
水样(海洋养殖系统中的生物曝气过滤系统) Water (biological aerated filter in a marine aquaculture system)	DM: KNO_3 1 g,$MgSO_4 \cdot 7H_2O$ 0.2 g,$CaCl_2$ 0.01 g,EDTA (0.5 mol/L) 0.5 mL,KH_2PO_4 0.5 g,Na_2HPO_4 0.5 g,$FeSO_4$ 0.01 g,NaCl 20 g,TE 溶液 5 mL NM: $(NH_4)_2SO_4$ 0.66 g,琥珀酸钠 1.35 g,$MgSO_4 \cdot 7H_2O$ 0.2 g,EDTA (0.5 mol/L) 0.5 mL,KH_2PO_4 0.5 g,Na_2HPO_4 0.5 g,TE 溶液 5 mL NDM: $NaNO_2$ 0.276 g,琥珀酸钠 3.16 g,$MgSO_4 \cdot 7H_2O$ 0.2 g,EDTA (0.5 mol/L) 0.5 mL,KH_2PO_4 0.5 g,Na_2HPO_4 0.5 g,$FeSO_4$ 0.01 g,NaCl 20 g,TE 溶液 5 mL	30 ℃、150 r/min	海杆菌(*Marinobacter* sp.)	异养硝化-好氧反硝化	[34]
海水(西太平洋) Sea water (Western Pacific ocean)	琥珀酸钠 4.72 g,KH_2PO_4 1 g,$MgSO_4 \cdot 7H_2O$ 1 g,$CaCl_2 \cdot 2H_2O$ 0.2 g,$FeSO_4 \cdot 7H_2O$ 0.05 g,$NaNO_2$ 0.002 5 g	30 ℃、180 r/min	蒙氏假单胞菌(*Pseudomonas monteilii*) CY06	好氧反硝化	[8]
水及泥样(虾池) Water and soil (shrimp pond)	琥珀酸钠 4.72 g,$NaNO_2$ 0.05 g,KH_2PO_4 1.5 g,Na_2HPO_4 0.42 g,$MgSO_4 \cdot 7H_2O$ 1 g	30 ℃、150 r/min	蜡样芽孢杆菌(*Bacillus cereus*) PB88	好氧反硝化	[60]
	$(NH_4)_2SO_4$ 0.5 g,KH_2PO_4 0.7 g,$MgSO_4 \cdot 7H_2O$ 0.5 g,$CaCl_2 \cdot 2H_2O$ 0.5 g,TE 溶液 1 mL	30 ℃、200 r/min	蜡样芽孢杆菌(*Bacillus cereus*) PB45	异养硝化-好氧反硝化	[61]
	琥珀酸钠 4.72 g,$NaNO_2$ 0.05 g,KH_2PO_4 1.5 g,Na_2HPO_4 0.42 g,$MgSO_4 \cdot 7H_2O$ 1 g	30 ℃、200 r/min	芽孢杆菌(*Bacillus* sp.) YX-6	好氧反硝化	[11]
	柠檬酸钠 8.5 g,KH_2PO_4 1 g,$MgSO_4 \cdot 7H_2O$ 1 g,$CaCl_2 \cdot 6H_2O$ 0.2 g, $FeCl_3 \cdot 6H_2O$ 0.05 g	32 ℃、120 r/min	海杆菌(*Marinobacter* sp.)	好氧反硝化	[62]

续表 1

样品(来源) Samples (source)	选择性培养基(每升含) Selective medium (per liter contained)	筛选条件 Screening conditions	菌株 Strains	脱氮类型 Denitrification mode	参考文献 Reference
沉积物(太湖) Sediment (*Taihu* lake)	KH_2PO_4 87.80 mg,$C_6H_5Na_3O_7 \cdot 2H_2O$ 3.26 g,NH_4Cl (NM) 0.382 g/$NaNO_2$(NDM) 0.493 g/KNO_3(DM) 0.722 g,$MgSO_4 \cdot 7H_2O$ 1.0 g,TE 溶液 2 mL	30 ℃、150 r/min	施氏假单胞菌(*Pseudomonas stutzeri*) YG-24	异养硝化-好氧反硝化	[63]
土样(粪便处理系统) Soil (nightsoil treatment system)	葡萄糖 1.05 g,NH_4Cl 0.382 g,KH_2PO_4 0.131 g,蛋白胨 0.05 g,酵母浸出物 0.05 g,TE 溶液 1 mL	30 ℃、150 r/min	芽孢杆菌(*Bacillus* sp.)	异养硝化-好氧反硝化	[64]
污泥(废水处理系统) Sludge (waste water treatment system)	$(NH_4)_2SO_4$ 0.5 g,琥珀酸钠 5.95 g,TE 溶液 50 mL	30 ℃、150 r/min	施氏假单胞菌(*Pseudomonas stutzeri*) YZN-001	异养硝化-好氧反硝化	[65]
污泥(盐碱湖) Sludge (saline-alkali lake)	NaCl 40 g,琥珀酸钠 4.73 g,$NaNO_3$ 0.86 g,KH_2PO_4 1.36 g,$(NH_4)_2SO_4$ 0.27 g,$MgSO_4 \cdot 7H_2O$ 0.19 g,TE 溶液 1 mL	30 ℃、180 r/min	盐田盐单胞菌(*Halomonas campisalis*)	异养硝化-好氧反硝化	[66]

HEPES:羟乙基哌嗪乙硫磺酸 hydroxyethyl piperazine ethylthiosulfonic acid;TE:微量元素 trace element;DM:反硝化作用培养基 denitrification medium;NM:硝化作用培养基 nitrification medium;NDM:亚硝酸盐降解培养基 nitrite denitrification medium;EDTA:乙二胺四乙酸 ethylenediamine tetraacetic acid。

表 2 反硝化反应中的催化酶及基因

Table 2 Enzymes and genes in denitrification reaction

反硝化反应 Denitrification reaction	催化酶 Enzymes	基因 Genes
$NO_3^-+2H^++2e^- \rightarrow NO_2^-+H_2O$	硝酸还原酶	*napA*、*napB*、*narGHI*
$NO_2^-+2H^++e^- \rightarrow NO+H_2O$	亚硝酸还原酶	*nirS*、*nirK*
$2NO+2H^++2e^- \rightarrow N_2O+H_2O$	一氧化氮还原酶	*norB*、*norC*
$N_2O+2H^++2e^- \rightarrow N_2+H_2O$	一氧化二氮还原酶	*nosZ*

由于亚硝酸盐对水产动物的毒害作用较为严重,催化亚硝酸盐转化的酶系非常值得深入研究。研究表明,亚硝酸盐可在由 *nir* 基因簇编码的亚硝酸盐还原酶、*nor* 基因簇编码的一氧化氮还原酶及 *nos* 基因簇编码的一氧化二氮还原酶等几种酶的催化下去除[67-68]。在好氧反硝化细菌中,主要存在 2 种类型的亚硝酸还原酶,一种以 CD1 血红素为辅因子,编码该酶的基因为 *nirS*;另一种以铜原子为辅因子,编码该酶的基因为 *nirK*,自然界中,*nirK* 基因分布的更广泛,但 *nirS* 基因更丰富。研究发现,这 2 种周质酶可以发挥相同的功能[69],但不能在同一种微生物中共存;然而,Sánchez 等[70]在慢生根瘤菌(*Bradyrhizobium oligotrophicum*)中发现了编码这 2 种酶的基因。Ma 等[8]对分离自虾池的好氧反硝化细菌蒙氏假单胞菌(*Pseudomonas monteilii*) CY06 全基因组测序确定了亚硝酸盐降解相关的 3 类还原酶,包括硝酸盐还原酶、亚硝酸还原酶、一氧化氮还原酶,通过这些酶菌株 CY06 能够实现反硝化过程($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$)的转化,并发现了 5 个亚硝酸还原酶相关的基因(*nir*1、*nir*2、*nir*3、*nir*4 及 *nir*5)。Huang 等[18]从海水养殖池塘中分离到一株高效脱氮 *Bacillus litoralis* N31,该菌株存在 *hao*、*napA* 和 *nirS* 基因,具备异养硝化-好氧反硝化能力。Zhang 等[71]对

分离自深海沉积物的菌株博尔扎诺假单胞菌(*Pseudomonas bauzanensis*)DN13-1 进行基因组分析,找到了同菌株脱氮性能相关的 *nirS*、*norB*、*nosZ*、*nasA* 和 *amo* 基因,证实了菌株存在好氧反硝化途径,其中 *nosZ* 基因已作为检测假单胞菌反硝化作用的生物标志物[72]。Wang 等[73]从假黄色单胞菌(*Pseudoxanthomonas*)基因组中扩增出 *nirS*、*nirK*、*narG* 和 *narA* 基因,该菌株同样具有好氧反硝化脱氮功能。然而,由于反硝化细菌的生物多样性,不同菌株的在降解过程起作用的关键酶和基因不同,且脱氮活性还与环境密切相关[74-75],这使得降解机制探索受到很大的限制,尚需深入研究。

5　应用研究

一般而言,体外筛选获得的好氧反硝化菌株还需要通过养殖动物应用试验评估,在实验室模拟水槽或养殖池塘进一步评价菌种的脱氮性能以及对水产动物生长性能、机体免疫和抗病性等方面的影响。应用实验室模拟水槽研究发现,好氧反硝化细菌 *Bacillus* sp. H2 对亚硝酸盐的平均降解率为 64.04%,总氮和化学需氧量的降解率分别为 16.0% 和 32.39%,且提升了水槽中鲤鱼的生长性能和免疫功能[76]。养殖池塘中的研究发现,均匀泼洒反硝化细菌 *Marichromatium gracile* YL28 显著降低了零换水对虾养殖池塘中氨氮、亚硝酸盐的积累[77],Gao 等[78]研究也发现,热带念珠菌(*Candida tropicalis*)HH8 和好氧反硝化菌株 *Pseudomonas stutzeri* LZX301 联合应用显示出更高效的脱氮效率,并能在池塘中快速形成生物菌落;Kong 等[79]发现除烃海杆菌(*Marinobacter hydrocarbonoclasticus*)是循环水产养殖系统好氧反硝化脱氮的潜在微生物。同时,还可借助于生物反应器以评估菌种的反硝化脱氮功能,Chen 等[5]选择好氧反硝化菌株 Z1 和 Z8 进行生物反应器脱氮试验,接种 2 周后达到相对稳定状态,该反应器硝酸盐去除率高于 98.8%,总氮去除率高于 71.8%,脱氮效果理想;Liu 等[80]研究发现,在好氧条件下,*Corynebacterium pollutisoli* 可在移动床生物反应器中可进行反硝化去除无机氮素。反硝化细菌饲喂后也能起到较好的脱氮效果,Thurlow 等[81]应用具有反硝化功能的贝莱斯芽孢杆菌(*Bacillus velezensis*)AP193 饲喂斑点叉尾鮰,显著提高动物生长性能,降低养殖池塘中总磷、总氮及硝酸盐浓度,改善水质环境。由此可见,在应用试验中,好氧反硝化菌种也表现出较高的脱氮效率,可改善养殖水体环境。然而,除菌种外,应用试验还受到养殖动物品种、应用剂量、应用方式及试验周期等方面因素的影响[82]。因此,应用养殖动物试验评价好氧反硝化菌种也需考虑上述几方面因素,同时还需结合菌种特点设计评价指标,从而对益生菌菌种功效进行综合评价。

6　小　结

综上所述,好氧反硝化细菌在养殖池塘的生物脱氮方面具有突出的优势与应用潜力,但不同菌种或相同菌种在不同条件下脱氮效率差别较大,可从样品采集、培养基配制、培养方式及评价方法等方面优选适合好氧反硝化细菌的方法、标准,进而实现好氧反硝化细菌菌种的快速、高效筛选。未来还需重点研究的内容包括以下几个方面:1)结合养殖水环境特点配制筛选培养基(碳源、氮源和碳氮比等)、设置筛选条件(溶氧、pH、温度和盐度等),筛选适合养殖水环境的好氧反硝化细菌;2)借助宏基因组测序技术,对养殖水体中反硝化脱氮菌群进行全面分析,从而挖掘更高效、耐受性更强的生物脱氮菌株;3)应用多组学技术(如基因组学、转录组学、蛋白质组学和代谢组学)从不同层次阐明好氧反硝化机理,比如蛋白质组学可用于研究反硝化酶活性中心的铁、铜等元素功能,通过代谢组学还可分析不同碳源在反硝化过程中关键代谢产物的变化;4)利用群体感应(quorum sensing,QS)的理论及技术,开展反硝化细菌之间或同其他益生菌的共培养、共发酵(co-culture)研究。不同微生物共存的菌系能够达到某种协同效应,可进一步提高脱氮效率,而且复合菌系还具有更强的适应性,建议对其深入研究并应用于水产养殖,从而实现更持久、更高效的养殖水体生物脱氮。

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Advances in Screening and Evaluation of Aerobic Denitrifying Bacteria in Aquaculture

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Abstract: High density farming techniques easily result in the accumulation of harmful nitrogen sources such as ammonia nitrogen and nitrite, which are highly toxic to water environments and cultured aquatic animals, and they do great harm to the aquaculture industry. However, aerobic denitrifying bacteria can use inorganic nitrogen and reduce it to these forms of reduced nitrate or reduce nitrogen, and then effectively improve water quality, and the strains screening is a key step in the research and application of aerobic denitrifying bacteria. In the review, we summarize the screening and evaluation methods for aerobic denitrifying bacteria, and discuss the methods and standards suitable for aquaculture through some aspects including sample collection, medium preparation, cultivation methods and evaluation methods. This paper will provide a reference for the screening and evaluation criteria for aerobic denitrifying bacteria in aquaculture, and make prospects for future research directions. [***Chinese Journal of Animal Nutrition*, 2021, 33(1): 20-32**]

Key words: aquaculture; aerobic denitrifying bacteria; screening methods

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