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耐冷嗜碱蒙氏假单胞菌 H97 的鉴定及其好氧反硝化 特性

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摘要:结合形态、磷脂脂肪酸及16S rRNA 基因序列分析鉴定了分离自贵州冬水田的好氧反硝化菌株 H97,采用模拟废水探讨了不同温度、接菌量、C/N、初始 pH 和碳源种类对菌株 H97 反硝化能力的影响.结果表明,菌株 H97 为蒙氏假单胞菌(Pseudomonas monteilii),目前国内外尚无蒙氏假单胞菌具有耐冷嗜碱好氧反硝化作用的研究报道,是对好氧反硝化菌的补充;该菌的最适脱氮条件为15℃、pH 9.0、C/N 15、接种量1.5×10⁶ CFU·(100 mL)⁻¹,碳源丁二酸钠.此外,菌株 H97 在 pH 为7.0~11.0 时,均表现出良好的脱氮能力,对硝酸盐氮和总氮的去除率分别可达 91.21% 和 79.10%以上,初始 pH 为 12.0 时,对硝酸盐氮和总氮的去除率仍分别达 64.75% 和 36.78%,表现出了较强耐碱能力.在初始硝酸盐氮浓度为 50.0 mg·L⁻¹和最适脱氮条件下,菌株 H97 在 48 h内对硝酸盐氮和总氮的去除率可达 97.69% 和 96.32%.同时,H97 对温度适应范围广,在 15~40℃均具有较强好氧反硝化能力,是 1 株耐冷嗜碱好氧反硝化细菌,在碱性氮污染水体处理中具有较好的应用潜力. 关键词:耐冷;嗜碱;蒙氏假单胞菌;脱氮;反硝化特性

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Identification and Characterization of a Hypothermic Alkaliphilic Aerobic Denitrifying Bacterium *Pseudomonas monteilii* Strain H97

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Abstract: Low temperatures and high pH generally inhibit bio-denitrification. Thus, it is important to explore psychrotrophic and alkali-resistant microorganisms for nitrogen degradation. This study mainly focused on the identification of an alkaliphilic strain and preliminary exploration of its denitrification characteristics. Based on morphological observations, phospholipid fatty acids and 16S rRNA gene sequence analysis, strain H97, which was isolated from the winter paddy field in Guizhou province, was identified as *Pseudomonas monteilii*. Till date, there were few reports about the denitrification characteristics of *Pseudomonas monteilii*. The effects of environmental factors such as temperature, inoculation quantity, C/N ratio, initial pH, and carbon source were investigated using simulated wastewater. The optimum conditions for nitrate and total nitrogen removal by H97 were; inoculum size 1.5×10^6 CFU·(100 mL)⁻¹; initial pH 9.0; C/N = 15; 15°C; and sodium succinate as the carbon source. The nitrate and total nitrogen removal efficiencies were 97.69% and 96.32%, respectively, at optimum conditions with an initial nitrate nitrogen concentration of 50.0 mg·L⁻¹. The temperature experiments indicated that the optimal temperature for highest nitrogen removal efficiency was 15°C, and that the strain H97 could survive in a wide range of 15-40°C. Additionally, the nitrate and total nitrogen efficiencies at the initial pH value of 7.0-11.0 were 91.21% and 79.10%, respectively, and the denitrification capacity then decreased to 64.75% at the initial pH 12.0. These results indicated that strain H97 showed cold and alkali resistance, which suggests an application potential for the treatment of alkaline nitrogen polluted water in the southern winter.

Key words: hypothermia; alkaliphilic; Pseudomonas monteilii; nitrogen removal; denitrification characteristics

好氧反硝化细菌因其具有适应性较强、生长速度快、容易控制等优点,而受到国内外研究者的青睐^[1].目前,已有大量好氧反硝化细菌的研究报道,其中以假单胞菌属最多^[2],主要有斯氏假单胞菌(Pseudomonas stutzeri)^[3]、铜绿假单胞菌(Pseudomonas aeruginosa)^[4]、托拉斯假单胞菌(Pseudomonas tolaasii)^[5]和恶臭假单胞菌(Pseudomonas putida)^[6]等.而国内外关于好氧反硝化蒙氏假单胞菌(Pseudomonas monteilii)的研究报

道很少,仅有张文艺等^[7]和冯雅丽等^[8]的研究报道.而张文艺等未报道该蒙氏假单胞菌 N1 的反硝化特性;冯雅丽等发现的蒙氏假单胞菌 YL-1 在 pH大于9.0,温度低于 25℃或高于 35℃时,基本丧失

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对硝酸盐氮的反硝化能力.

事实上,脱氮生物菌易受外在环境影响,环境中的温度和酸碱性就是影响生物脱氮的重要因素.目前报道的脱氮微生物大多数是在 20~35℃、中性或微碱性条件下进行,对低温、过酸或过碱的环境条件耐受性较弱. 如菌株 Pseudomonas stutzeri F1^[9]在温度低于 20℃或 pH 值大于 8.0 的条件下,其生长与反硝化脱氮作用均受到抑制;菌株 Pseudomonas sp. 2-8^[10]在温度低于 20℃或 pH 值大于 9.0 的条件下,基本丧失反硝化能力. 而现实中碱性氮污染废水的来源广泛,如生产化肥的废水 pH 值达 8.0~10.0^[11];生产石油树脂的废水 pH 值可达 11.0~13.0^[12]. 因此,研究耐冷嗜碱高效生物脱氮菌对处理南方地区冬季碱性废水中的氮污染具有重要意义.

本实验从贵州冬水田中分离获得 1 株具有好氧 反硝化作用的菌株 H97,结合形态学、磷脂脂肪酸 及 16S rRNA 基因序列分析,该菌被鉴定为蒙氏假 单胞菌(Pseudomonas monteilii),通过环境因子对菌 株生物脱氮的影响发现,该菌在 15~40℃和 pH 7.0~12.0 范围内均能进行高效的脱氮作用,研究结果 为南方地区冬季碱性废水中氮污染生物处理提供了一种选择,以期为此后该菌的具体应用奠定基础.

1 材料与方法

1.1 材料

1.1.1 菌株来源

新分离菌株 H97 为本实验室从贵州省冬水田中分离筛选获得.

1.1.2 培养基

- (1) BTB 培养基(bromothymol blue, BTB)^[13]: NaNO₂ 1.0 g·L⁻¹, KH₂PO₄ 1.0 g·L⁻¹, FeCl₂·6H₂O 0.5 g·L⁻¹, CaCl₂·7H₂O 0.2 g·L⁻¹, MgSO₄·7H₂O 1.0 g·L⁻¹, 琥珀酸钠 8.5 g·L⁻¹(固体加 2.0% 的琼脂),BTB(1.5% 溴百里酚蓝溶于无水乙醇)1mL,调节 pH 至 7.2.
- (2) 反硝化培养基(DM)^[14]: 乙酸钠 2.56 g·L⁻¹, NaNO₃ 0.31 g·L⁻¹, KH₂PO₄ 1.5 g·L⁻¹, Na₂HPO₄ 0.42 g·L⁻¹, MgSO₄·7H₂O 1.0 g·L⁻¹, FeSO₄·7H₂O 0.05 g·L⁻¹, 调节 pH 至 7.2.
- (3) LB 培养基: 胰蛋白胨 10.0 g·L⁻¹, 酵母粉 5.0 g·L⁻¹, NaCl 10.0 g·L⁻¹, 固体加 2.0% 的琼脂, 调节 pH 至 7.2.

将上述所有培养基在 0.11 MPa、121℃条件下 灭菌 30min,冷却后备用.

1.2 细菌鉴定

1.2.1 形态学特征观察

将分离纯化的菌株 H97 于 BTB 固体培养基上划线,在15℃条件下培养,待长出菌落后观察其菌落形态,采用 AFM 原子力显微镜(DIMENSION ICON WITH SCANASYST,美国 BRUKER 公司)扫描观察菌体形态.

1.2.2 特异性磷脂脂肪酸鉴定

取纯化菌株 H97 进行皂化、甲基化、萃取以及碱洗涤后获得上机样品,用 Agilent 6850 气相色谱仪(FID 检测器)分析特异性磷脂脂肪酸(Phospholipid fatty acid, PLFA)的成分. 各脂肪酸成分通过 MIDI Sherlock 微生物鉴定系统(Version6.1, MIDI, Inc., Newark, DE)进行检测.

1.2.3 菌株 H97 的 16S rRNA 基因序列检测

以纯化菌株 H97 的基因组 DNA 为模板,采用通用引物 27F(5'-AGAGTTTGATCCTGGCTCAG-3')和 1492R(5'-GGTTACCTTGTTACGACTT-3')进行16S rRNA 聚合酶链式反应(PCR)扩增,将克隆产物送测序公司测序,将 16S rRNA 基因序列在 GenBank核酸序列数据库中进行同源性比较,以 Neighbor-Joining 法^[15]构建系统发育树.

1.3 影响菌株 H97 好氧反硝化特性的因素分析

向装有 100 mL 反硝化培养基的 250 mL 三角瓶中接入菌株 H97,研究不同温度(5、10、15、20、25、30、35 和 40°C)、接菌量[0.5×10^6 、 1.0×10^6 、 1.5×10^6 、 2.0×10^6 和 2.5×10^6 CFU·(100 mL) $^{-1}$]、C/N 值(0、5、10、15、20 和 25)、初始 pH 值(6.0、7.0、8.0、9.0、10.0、11.0、12.0 和 13.0)和碳源种类(柠檬酸钠、丁二酸钠、葡萄糖、蔗糖和乙酸钠)对菌株 H97 反硝化特性的影响。以上处理均在 150 r·min $^{-1}$ 摇床条件下培养 48 h 后,测定菌株 H97 的生长、硝酸盐氮和总氮的浓度,并计算硝酸盐氮和总氮的去除率.

1.4 分析方法

本研究中,硝酸盐氮的测定采用紫外分光光度法 $^{[16]}$,总氮的测定采用碱性过硫酸钾消解紫外分光光度法 $^{[17]}$,菌株的生长采用光度比浊法(D_{600}). 脱氮率计算方法如下:

$$R = \left[1 - \left(\frac{c_t}{c_0}\right)\right] \times 100\% \tag{1}$$

$$V = (c_0 - c_t)/t \tag{2}$$

式中,R 为氮的去除率, c_l 为终态氮浓度($mg \cdot L^{-1}$), c_0 为初始氮浓度($mg \cdot L^{-1}$),V 为氮的去除速率 [$mg \cdot (L \cdot h)^{-1}$],t 为时间.

采用 Excel、SPSS Statistics 19.0、MEGA 6.0 和

Origin 8.6 软件对实验数据进行统计分析与作图.

2 结果与分析

2.1 细菌鉴定

2.1.1 形态学特征

在 BTB 固体培养基上观察到菌株 H97 的菌落 形态特征为:圆形,呈黑色,表面湿润光滑,边缘整 齐,中央略凸起,革兰氏染色为阴性. 经原子力显微 镜扫描观察,结果如图 1 所示,菌体呈短杆状或椭球 状,不产芽孢,无鞭毛.

2.1.2 特异性磷脂脂肪酸鉴定

磷脂脂肪酸(PLFAs)是微生物细胞膜的主要成分,不同类群微生物细胞膜的 PLFAs 存在差异,同种类群微生物 PLFAs 含量比较恒定^[18],它的种类和含量是细胞分类法的重要依据之一. 气相色谱能快速、灵敏、精确地检测出细菌细胞内的脂肪酸^[19].

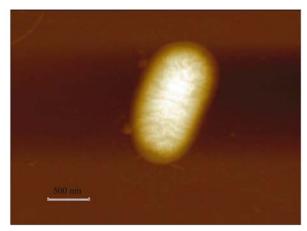


图 1 原子力显微镜扫描下的菌株 H97

Fig. 1 Strain H97 under the scanning atomic force microscope

本研究中,菌株 H97 的磷脂脂肪酸鉴定结果如表 1 所示,该菌株与 *Pseudomonas syringae* tomato 的相似性为 0.569.

表 1 特异性磷脂脂肪酸的鉴定结果

| | Table 1 Result of the specific pl | hospholipid fatty acid identification | 3/2 |
|-------------|-----------------------------------|---------------------------------------|--------|
| 数据库 | 相似指数 | 菌种名称 | (6) |
| RTSBA66. 21 | 0. 569 | Pseudomonas syringae tomato | (//) |
| | 0. 444 | Pseudomonas fluorescens biotype A | 2/1 |
| | 0. 432 | Pseudomonas syringae maculicola | 4.00 |
| 0 /01 | 0. 423 | Pseudomonas syringae syringae | |
| 6 6 611 | 0.377 | Photobacterium angustum | ~ |
| 7/2// | 0. 368 | Pseudomonas fluorescens biotype C/P | Ja 5 5 |
| 1166 | 0.444 | Pseudomonas fluorescens biotype A | |
| C 30 1/0 | 0. 432 | Pseudomonas syringae maculicola | 1 |
| CR VI TO | 0. 423 | Pseudomonas syringae syringae | |
| 9 10 1 | 0. 377 | Photobacterium angustum | |
| RCLIN66. 20 | 0. 359 | Neisseria cinerea GC subgroup B | |
| M. | 0. 300 | Chromobacterium violaceum | |
| 4 | 0. 286 | Aeromonas veronii GC subgroup B | |
| | 0. 271 | Pseudomonas putida biotype A | |
| | 0. 232 | Arcobacter cryaerophilus | |
| MI7H103. 80 | | (没有匹配) | |

2.1.3 16S rRNA 基因序列分析

菌株 H97 的 16S rRNA 基因全长1 392 bp, 在

GenBank 中的登录号为 KY927410,系统发育树如图 2 所示,该菌与 Pseudomonas monteilii 的同源性达

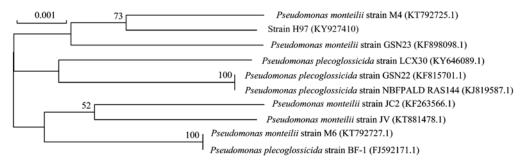


图 2 菌株 H97 的系统发育进化树

Fig. 2 Phylogenetic tree of strain H97

99%. 基于磷脂脂肪酸鉴定的结果,在传统分类学上, Pseudomonas monteilii 属于丁香假单胞菌(Pseudomonas syringae)^[20],这与该菌 16S rRNA 基因序列分析的结果一致. 因此,结合形态学、磷脂脂肪酸和 16S rRNA 基因序列分析的结果,可以将菌株 H97 确定为蒙氏假单胞菌(Pseudomonas monteilii).

2.2 影响菌株 H97 好氧反硝化特性的因素分析

2.2.1 温度对菌株 H97 反硝化特性的影响

如图 3 所示, 当温度为 5℃和 10℃时, 菌株 H97 几乎不能生长; 但当温度上升到 15℃时, 48 h 内, 硝酸盐 氮浓度由 52.73 mg·L⁻¹ 下降至 11.02 mg·L⁻¹, 平均反硝化速率为 0.87 mg·(L·h)⁻¹, 总氮的平均去除速率为 0.81 mg·(L·h)⁻¹, 菌株 H97 对硝酸盐氮和总氮的去除率分别达 80.55% 和73.18%; 当温度为 15~30℃范围内时, 菌株 H97的生长和脱氮效率基本保持不变; 当温度继续上升至 40℃时, 菌株 H97的生长和脱氮率逐渐降低, 对硝酸盐氮和总氮的去除率分别为 63.60%和48.65%.

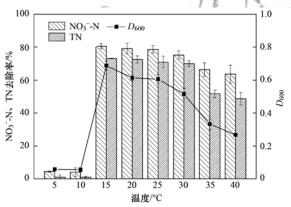


图 3 温度对菌株 H97 反硝化特性的影响

Fig. 3 Effects of temperature on the denitrification ability of strain H97

2.2.2 接菌量对菌株 H97 反硝化特性的影响

如图 4 所示, 当接菌量为 0.5×10^6 CFU 时(每 100 mL 培养基), 硝酸盐氮和总氮的去除率仅为 18.01% 和 17.23%;但当接菌量上升至 1.5×10^6 CFU·(100 mL) $^{-1}$ 时, 48 h 内, 硝酸盐氮浓度由 48.46 mg·(L·h) $^{-1}$ 下降至 5.76 mg·(L·h) $^{-1}$, 平均反硝化速率为 0.89 mg·(L·h) $^{-1}$, 总氮的平均去除速率为 0.68 mg·(L·h) $^{-1}$, 菌株 H97 对硝酸盐氮和总氮去除率分别达 88.12% 和 59.07%;将接菌量继续增大到 2.5×10^6 CFU·(100 mL) $^{-1}$ 时, 总氮的去除率下降到 57.82%. 因此, 在实际应用中可以将

菌株 H97 的接菌量控制在 $1.5 \sim 2.0 \times 10^6$ CFU·(100 mL)⁻¹.

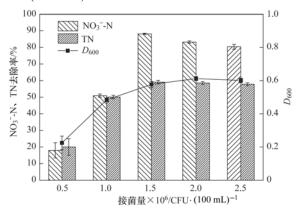


图 4 接菌量对菌株 H97 反硝化特性的的影响

Fig. 4 Effects of inoculation quantity on the denitrification ability of strain H97

2.2.3 C/N 对菌株 H97 反硝化特性的影响

如图 5 所示,当 C/N 小于 10 时,菌株 H97 的生长和反硝化活性受到一定程度的抑制,对硝酸盐氮的去除不完全,几乎不能去除总氮;当 C/N 上升至15 时,硝酸盐氮浓度由 50.93 $\mathrm{mg}\cdot\mathrm{L}^{-1}$ 下降至 0.44 $\mathrm{mg}\cdot\mathrm{L}^{-1}$,平均反硝化速率为 1.05 $\mathrm{mg}\cdot(\mathrm{L}\cdot\mathrm{h})^{-1}$,总氮的平均去除速率为 0.7 $\mathrm{mg}\cdot(\mathrm{L}\cdot\mathrm{h})^{-1}$,该菌对硝酸盐氮和总氮的去除率分别达 99.13% 和 61.42%;当 C/N 大于 15 时,脱氮率呈下降趋势.综合考虑,菌株 H97 的最佳 C/N 为 15.

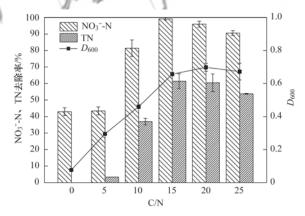


图 5 C/N 对菌株 H97 反硝化能力的影响

Fig. 5 Effects of C/N on the denitrification ability of strain H97

2.2.4 初始 pH 对菌株 H97 反硝化特性的影响

如图 6 所示,当 pH 为 6.0 时,不利于菌株 H97 的生长;当 pH 7.0~11.0 时,菌株 H97 能很好生长,对硝酸盐氮和总氮的去除率较高,尤其 pH = 9.0 时,硝酸盐氮浓度由 50.92 $mg \cdot L^{-1}$ 下降到 0.47 $mg \cdot L^{-1}$,平均反硝化速率为 1.05 $mg \cdot (L \cdot h)^{-1}$,总氮由 53.44 $mg \cdot L^{-1}$ 下降至 4.39 $mg \cdot L^{-1}$,平均去除

速率 $1.02 \text{ mg} \cdot (\text{L} \cdot \text{h})^{-1}$,该菌对硝态氮和总氮的去除率分别达 99.07% 和 91.78%; pH = 11.0 时,菌 株 H97 生长较好,脱氮效果较强; pH = 12.0 时,其 生长变弱,仍具有一定脱氮能力,对硝酸盐氮和总氮的去除率分别为 64.75% 和 36.78%.

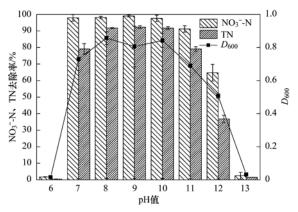


图 6 pH 对菌株 H97 反硝化能力的影响

Fig. 6 Effects of pH on the denitrification ability of strain H97

2.2.5 碳源种类对菌株 H97 反硝化特性的影响

如图 7 所示,碳源种类对菌株 H97 的生长及脱氮率的影响存在很大差异. 当以柠檬酸钠、丁二酸钠和乙酸钠为碳源时,菌株 H97 均能很好地生长,尤其以丁二酸钠为碳源时,48 h内,硝酸盐氮浓度由 50.40 mg·L⁻¹下降至 1.17 mg·L⁻¹,平均反硝化速率达 1.03 mg·(L·h)⁻¹,总氮由 55.53 mg·L⁻¹下降至 2.05 mg·L⁻¹,平均去除速率为 1.11 mg·(L·h)⁻¹,其对硝酸盐氮和总氮的去除率分别达97.69%和 96.32%;当以葡萄糖为碳源时,其对总氮的去除率仅为 46.42%;而以蔗糖为碳源时,其对总氮的去除率仅为 46.42%;而以蔗糖为碳源时,该菌几乎不能生长.因此,综合考虑菌株 H97 的生长和脱氮效果,以及丁二酸钠价格昂贵,在实际污水处理

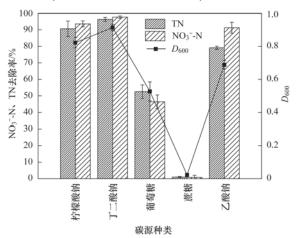


图 7 碳源种类对菌株 H97 反硝化能力的影响

Fig. 7 Effects of carbon source on the denitrification ability of strain H97

中可以将柠檬酸钠或乙酸钠作为菌株 H97 的最佳 碳源.

3 讨论

温度是影响微生物反硝化特性的重要因素之一,主要是通过影响微生物细胞结构来影响其生长和新陈代谢^[21]. 一般地,好氧反硝化细菌的最适温度为 20~35℃,如 Ye 等^[22]报道了菌株 Providencia rettgeri strain YL 的最适脱氮温度为 25℃;李卫芬等的研究表明^[23],菌株 Pseudomonas stutzeri F1 在温度高于 35℃时,其生长和脱氮能力均受到抑制. 上述结论与本研究结果不同,菌株 H97 能够适应 15~40℃的环境温度,优于目前分离出的大多数好氧反硝化细菌;该菌在 15℃时生长最好,脱氮率最高.这可能是因为该菌分离自贵州长期淹水的冬水田泥土中,在 15℃实验条件下分离筛选获得,对低温环境的适应能力较强,在低温环境下能较好地进行脱氮作用. 因此,该菌为处理南方地区冬季氮污染水体提供了选择.

接菌量是影响细菌反硝化特性的重要因素之一. 在反硝化过程中, 若接菌量不足则不能充分发挥微生物生长繁殖的潜力, 导致脱氮效果差; 而接菌量过多会导致细菌对营养物质的竞争, 部分细菌死亡, 脱氮率降低. He 等的研究发现^[24], 当接菌量为 1.5 × 10⁶ CFU 时, 好氧反硝化细菌 Arthrobacter arilaitensis strain Y-10 对总氮的去除率最高(52.5%), 当接菌量上升至 2.5 × 10⁶ CFU 时, 总氮的去除率下降至 32.27%, 与本研究结果相似.

C/N 是菌株能否进行完全反硝化的关键因素,碳氮源的比例直接影响细菌的生长和对有机物质的去除效果.若初始碳源充足,则能为细菌脱氮提供足够的能量;若碳源不足,则不能提供足够的能量供细菌生长,导致反硝化效率降低[25].但当碳源高于细菌的需求时,其生长和脱氮率会保持在一个稳定水平,若继续增加碳源,脱氮效率不会有太大变化,甚至有下降趋势[26]. Duan等的研究发现[27],菌株 Vibrio diabolicus SF16 对氮的去除率首先随 C/N值的增大而升高,当 C/N 达 10 时去除率最高(93.00% ±2.31%),之后随 C/N值的增大而降低.菌株 H97 的最佳 C/N为 15,但当 C/N为 10 时,该菌依然可以表现出良好的脱氮效果,因此,在实际应用过程中,可通过延长生物脱氮时间,而达到良好的脱氮效果.

一般来说,过酸或过碱都会影响细菌的酶活性,

导致反硝化能力降低. 目前分离出的大多数好氧反硝化细菌最适 pH 为中性或偏碱性,在 pH = 11.0 时几乎不能生长,不表现反硝化活性或反硝化活性很低. Wang 等^[28]对好氧反硝化假单胞菌 HS-N62 的研究表明,当 pH 达 10.0 时,其对硝酸盐氮的去除率不足 50%,与本研究结果不同,菌株 H97 能在 pH = 11.0 的碱性条件下高效脱氮, pH = 12.0 时,仍能生存和脱氮,具嗜碱特性,表明菌株 H97 对处理碱性环境中的氮污染水体具有应用价值.

在反硝化过程中,碳源作为电子供体和供微生物生长繁殖所需的营养物质,最终将硝酸盐氮还原成含氮气体,达到除氮效果^[29].不同碳源在微生物代谢过程中的地位不同,菌株对其的利用率也不同^[30].在本研究中,菌株 H97 对柠檬酸钠、丁二酸钠和乙酸钠的利用率较高,对葡萄糖的利用率较低,几乎不能利用蔗糖.可能是由于结构越简单,分子量越小的碳源越容易被微生物降解,而且柠檬酸钠、丁二酸钠和乙酸钠是三羧酸循环的中间产物,更容易被菌株 H97 利用^[31].

4 结论

- (1)本实验从贵州冬水田中分离获得1株具有好氧反硝化作用的菌株 H97,结合形态学、磷脂脂肪酸及16S rRNA 基因序列分析,该菌被鉴定为蒙氏假单胞菌(Pseudomonas monteilii).
- (2) 菌株 H97 具有较强好氧反硝化能力, 在初始硝酸盐氮为50 mg·L⁻¹时, 其最佳脱氮条件为: 温度 15℃, 接菌量为 1.5×10^6 CFU·(100 mL)⁻¹, pH 9.0, C/N = 15, 碳源丁二酸钠.
- (3)菌株 H97 能在 15~40℃ 范围内生存和脱氮,具有一定的耐冷特性,对环境温度适应范围较广;在 pH = 12.0 时,仍能生长和脱氮,具嗜碱特性,表明菌株 H97 是 1 株耐冷嗜碱好氧反硝化细菌.参考文献:
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