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The potential of the acetonitrile biodegradation by *Mesorhizobium* sp. F28

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ABSTRACT

Mesorhizobium sp. F28 was used in the NHase/amidase enzyme system to convert acetonitrile into acetamide and acetic acid, and the cells grew with the production of acetic acid. The NHase activity of the strain F28 was 78 U mg^{-1} dcw, observed in the conversion of 19.5 mM acetonitrile at 0.2 h. As the initial pH value was between 6.5 and 8.3, 18.3 mM acetonitrile completely converted into acetamide within 2 h and the accumulation of acetamide subsequently converted into acetic acid and ammonia within 46 h. When 20.3 mM acetamide was added in the medium, the conversion rate of acetonitrile was 80% at 2 h and the conversion rate of the accumulative acetamide was slightly affected. The concentrations of acetic acid and ammonia were respectively 6.01 and 6.68 mM at 46 h. The addition of acetic acid decreased the activities of the NHase and amidase. The conversion rate of acetonitrile was 94% at 9.5 h and traces of acetic acid (0.25 mM) and ammonia (0.29 mM) were produced. The effects of product-inhibition indicated that the appropriate operation of bioreactor would be beneficial for *Mesorizobium* sp. F28 to degrade acetonitrile continuously.

1. Introduction

Acetonitrile is widely used in the industries as perfumes, rubber products, pesticides, or used to make pharmaceuticals. It is also applied as a mobile phase for HPLC analyses, or as a solvent to extract fatty acids from animal and vegetable oils [1,2]. The efficient process for acetonitrile removal should be designed to avoid the risks of waste discharging into the natural environment since acetonitrile is a toxic chemical that may cause severe health effects [3]. The microbial process is of interest to use in the detoxification of acetonitrile-containing waste. The packed-bed biofilm reactor equipped with an external aeration chamber for acetonitrile removal under aerobic conditions has been designed to investigate the treatment of acetonitrile HPLC waste [4]. The biodegradation of acetonitrile into ammonia and acetic acid has been developed, and the conversion efficiencies were 92–100% as the organic load was 2 g acetonitrile l^{-1} d^{-1} [1]. The biodegradation of acetonitrile of the adapted mixed culture in the batch reactor shows a two-step pathway with the generation of acetamide followed by acetic acid and ammonia [5]. For complete removal of acetonitrile in the bioprocess, microorganisms which can efficiently degrade acetonitrile are of considerable importance, and the proper operation must be determined. The strains, *Nocardia rhodochrous* LL100-21 [6], *Pseudomonas putida* [3], *Chromobacterium* sp., *Pseudomonas aeruginosa* [7], *Geotrichum* sp. JR1 [8], and *Rhodococcus* sp. RHA1 [9] can use acetonitrile as the sole carbon and nitrogen source. *Mesorhizibium* sp. F28, which was isolated from the nitrile-polluted wastewater, contains the nitrile hydratase (NHase, EC 4.2.1.84)/amidase (EC 3.5.1.4) enzyme system to efficiently convert acrylonitrile [10]. In this research, the biodegradation of acetonitrile by *Mesorhizibium* sp. F28 was investigated. Moreover, the optimum pH and the product-inhibitory effects for acetonitrile removal had been examined to realize the suitable operational process.

2. Materials and methods

2.1. Organisms, media and culture conditions

Mesorhizobium sp. F28 (GenBank accession number EU350515) was isolated from a wastewater treatment system in a polyacrylonitrile (PAN) fibre factory in Taiwan. The bacterium was maintained by transferring colonies at approximately monthly intervals on R2A agar [11] stored at 4 °C. For the preparation of resting cells, single colonies from R2A agar plate stock cultures were subcultured at 30 °C and shaken at 120 rpm in R2A liquid medium. The preculture was inoculated into R2A liquid medium containing 0.01 g l^{-1} $CoCl_2 \cdot H_2O$. The medium incubated at 30 °C

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for 24 h under aeration and harvested by centrifugation (6000 × *g* for 12 min). The harvested cells were washed with the phosphate buffered medium (PBM, pH 7.5) [12,13], and recentrifugated under the same conditions. The cell pellets were used as resting cells.

2.2. Acetonitrile biodegradation

To observe acetonitrile degradation by *Mesorhizobium* sp. F28, a series of batch experiments were conducted in 120 ml serum bottles. Each serum bottle containing cells suspended in 40 ml phosphate buffer medium (PBM, pH 7.5) to a cell concentration of 10^7 cell ml $^{-1}$, and then various concentrations of acetonitrile (2.32, 4.20, 9.34, 12.1, 18.3, and 19.5 mM) were added to the medium. After sealing with teflon/silicon stoppers, the reactors were shaken at 120 rpm in the dark at 30 °C. The concentrations of acetonitrile, acetamide, acetic acid and ammonia were analyzed at regular intervals. The value of pH and O.D.$_{600}$ were also observed in the experiment. One unit (U) of NHase activity was defined as the amount of resting cells that catalyses 1 μmol of acetonitrile per min under the specified conditions.

2.3. The optimum pH for acetonitrile biodegradation

The same process of the batch experiments was used except that the pH value of the PBM. Various pH values of phosphate buffer medium (pH 4.2, 5.5, 6.5, 7.5, 8.3), which were adjusted with NaOH/HCl, were adjusted to investigate the optimum pH for acetonitrile biodegradation. Acetonitrile (18.3 mM) was added to observe its biodegradation by *Mesorhizobium* sp. F28.

2.4. The effect of product-inhibiton on the acetonitrile biotransformation

To investigate the effect of acetamide and acetic acid on the acetonitrile biotransformation by *Mesorhizobium* sp. F28, the same process of the batch experiment was conducted. Each serum bottle (40 ml PBM, pH 7.5), which contained cell suspension (10^7 cell ml^{-1}) and acetonitrile (15.4 mM), was supplemented with acetamide (12.4, 16.4, and 20.3 mM) or acetic acid (12.8, 16.5, and 21.6 mM). The reactors were shaken at 120 rpm in the dark at 30 °C to observe acetonitrile biodegradation.

2.5. Analytical methods and chemicals

Samples were collected directly from the reactors using a syringe and then filtered (Millex GV-Filter, 0.22 μm pore size, Millipore). Acetonitrile, acetamide, and acetic acid were determined by a gas chromatograph (GC) equipped with a flame ionization detector. GC was performed with a HP 6890 system equipped with an Agilent DB-WAXETR column (1.0 μm × 30 m, 0.35 mm I.D.; USA). The injector and detector temperatures were both set at 250 °C. The column temperature was 100 °C for the initial 10 min after injection, followed by a temperature ramp at 6 °C min^{-1} up to 180 °C. Nitrogen gas was supplied as the carrier gas and the flow rate was 3.0 ml min^{-1}. The ammonia concentration was measured using the indophenol blue method [14]. The pH and O.D.$_{600}$ were measured respectively using a pH meter and Spectrophotometer (HITACHI U2800). All chemicals were from commercial sources and of analytical grade.

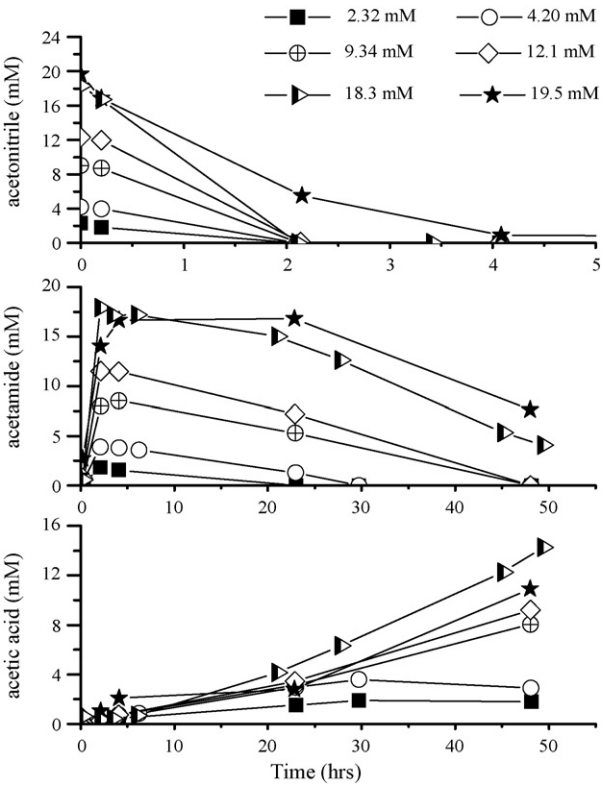

Fig. 1. The biodegradation of acetonitrile by *Mesorhizobium* sp. F28. Various concentrations of acetonitrile (2.32, 4.20, 9.34, 12.1, 18.3, and 19.5 mM) were added to the phosphate buffer medium (PBM, pH 7.5). The reaction was carried out at 30 °C with 120 rpm shaking.

3. Results and discussion

3.1. The biodegradation of acetonitrile by Mesorhizobium sp. F28

Fig. 1 shows the results of acetonitrile in different initial concentrations (2.32, 4.20, 9.34, 12.1, 18.3, and 19.5 mM) being converted into acetamide and acetic acid by the strain F28. Complete acetonitrile removal was achieved within 2 h when the initial concentration of acetonitrile was lower than 18.3 mM. The accumulation of acetamide gradually converted into acetic acid and ammonia by the amidase of *Mesorhizobium* sp. F28. As the acetonitrile concentration reached to 19.5 mM, this strain completely converted acetonitrile into acetamide and acetic acid within 4 h. The maximum NHase activity (78 U mg^{-1} dcw) was observed in the conversion of 19.5 mM acetonitrile at 0.2 h. Though the high concentrations of acetonitrile (2.32, 4.20, 9.34, 12.1, 18.3, and 19.5 mM) were added, the high accumulation of acetamide (1.83, 3.90, 8.54, 11.5, 17.9, and 16.8 mM) still efficiently converted into acetic acid and ammonia. At the end of the reaction, the acetic acid concentrations were 1.81, 3.60, 8.05, 9.20, 14.2, and 10.9 mM, respectively. The ammonia concentrations were 2.54, 4.50, 7.60, 8.58, 11.4, and 8.83 mM, respectively. During the experiment period, the O.D.$_{600}$ values increased obviously as the acetic acid was produced (data not shown).

The results of the acetonitrile biodegradation by *Pseudomonas putida* indicated that the pH increased rapidly from 7.0 to 8.9 with the high accumulation of ammonia (60.0 mM) and low production of acetic acid which was initially produced to 3.80 mM while disappeared after 36 h of incubation [3]. As *Pseudomonas aeruginosa* converted acetonitrile into acetic acid and ammonia, the pH increased from 7.0 to 8.9 since the ammonia concentration

accumulated to 37.0–38.0 mM and the highest acetic acid concentration was just 700×10^{-4} mM which might attribute to rapid utilization of acetic acid [15]. The acetonitrile biodegradation by nitrile-degradation isolates exhibited that the pH values increased from 7.0 to approximately 8.4 while the ammonia accumulated above 12.0 mM. The acetic acid concentration was just reached to 4.00 mM and decreased gradually to 0 mM after a period of reaction since acetic acid served as the carbon energy source for growth [16]. On the other hand, the pH values dropped slightly as *Mesorhizobium* sp. F28 converted acetonitrile into acetic acid and ammonia. The previous research has indicated that the decrease of the pH values in the glycolysis was due to the production of acetic acid [17]. Thus, acetic acid and ammonia which were the products of acetonitrile biodegradation might affect the variation of the pH values. In this study, the acetic acid concentrations were similar with or higher than the ammonia concentrations. Moreover, the acetic acid did not decrease rapidly during the reaction. From above, it suggested that the pH values would increase as the ammonia concentration was higher than the acetic acid concentration and decrease in the opposite situation.

Kohyama et al. have mentioned that the conversion of acetamide to acetic acid by *R. pyridinivorans* S85-2 was much difficult to proceed compared with that of acetonitrile to acetamide. The two-step process combined with *R. pyridinivorans* containing NHase and *B. diminuta* AM10-C-1 containing amidase was designed to entirely convert acetonitrile [18]. On the other hand, *Mesorhizobium* sp. F28 efficiently converted acetonitrile into acetic acid without adding other bacteria, and used acetic acid as a substrate to grow. The strain F28 has potential to degrade higher concentrations of acetonitrile, and further experiments could be conducted.

3.2. The optimum pH for acetonitrile biodegradation

The optimum pH for *Mesorhizobium* sp. F28 to convert acetonitrile was determined by using phosphate buffer (PBM) with different initial pH values (Fig. 2). The results exhibited that 18.3 mM acetonitrile converted completely into acetamide and acetic acid within 2 h though the initial pH values ranged from pH 4.2 to 8.3. It was noticed that different pH values affected the conversion of acetamide into acetic acid. As the initial pH value was 4.2 and 5.5, the residual concentrations of acetamide were 16.0 and 9.50 mM, respectively. The acetic acid concentrations were 2.08 and 9.03 mM, respectively. The ammonia concentrations were 2.34 and 8.89 mM, respectively. While the initial pH value was above 6.5, *Mesorhizobium* sp. F28 efficiently converted acetamide into acetic acid and ammonia. The concentrations of acetamide, acetic acid, and ammonia were respectively 3.00, 15.0, and 12.6 mM at the end of reaction when the initial pH value was 6.5. It was obvious that amidase activity of *Mesorhizobium* sp. F28 would decrease in a low pH medium. Therefore, the optimum pH for acetonitrile degradation by the strain F28 was between 6.5 and 8.3. The previous research has demonstrated that the desired products could be obtained by controlling reaction conditions as using NHase/amidase enzyme system of *Microbacterium imperiale* [19]. It suggested that the enzyme activities of *Mesorhizobium* sp. F28 might be regulated by reaction conditions.

3.3. The effect of acetamide on the acetonitrile biodegradation

Since *Mesorhizobium* sp. F28 used the NHase/amidase enzyme system to convert acetonitrile into acetamide and acetic acid, the effect of the products on the acetonitrile biodegradation was examined. The biodegradation of 15.4 mM acetonitrile as adding various concentrations of acetamide was exhibited in Fig. 3. When the medium were supplemented with 0, 12.4, 16.4, and 20.3 mM

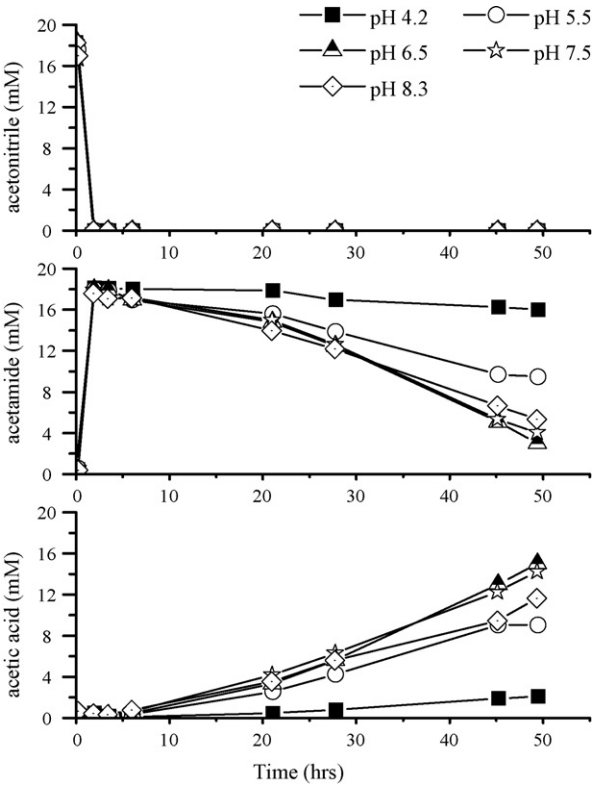

Fig. 2. The conversion of 18.3 mM acetonitrile by *Mesorhizobium* sp. F28 in a batch reactor with different initial pH values (pH 4.2, 5.5, 6.5, 7.5, 8.3). The reaction was carried out at 30 °C with 120 rpm shaking.

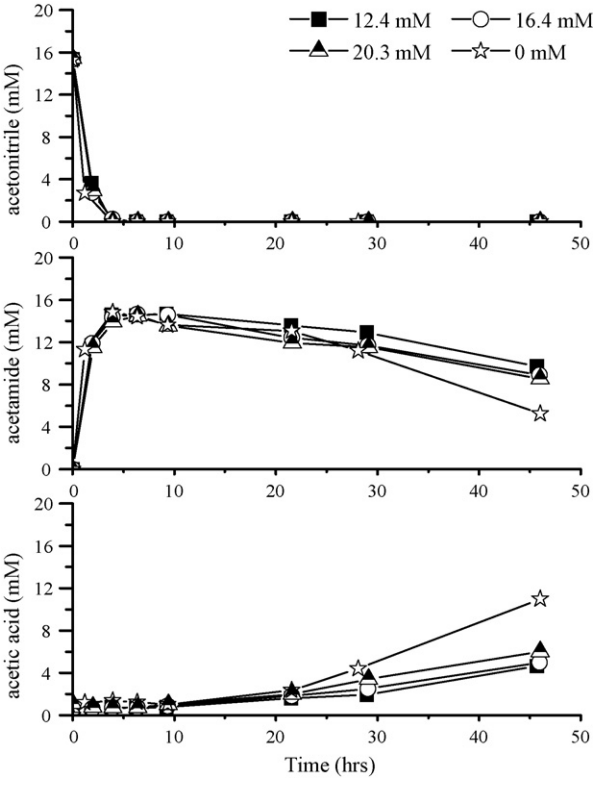

Fig. 3. The conversion of 15.4 mM acetonitrile by *Mesorhizobium* sp. F28 in a batch reactor containing the phosphate buffer medium (pH 7.5) supplementation with 12.4, 16.4, and 20.3 mM acetamide. The reaction was carried out at 30 °C with 120 rpm shaking.

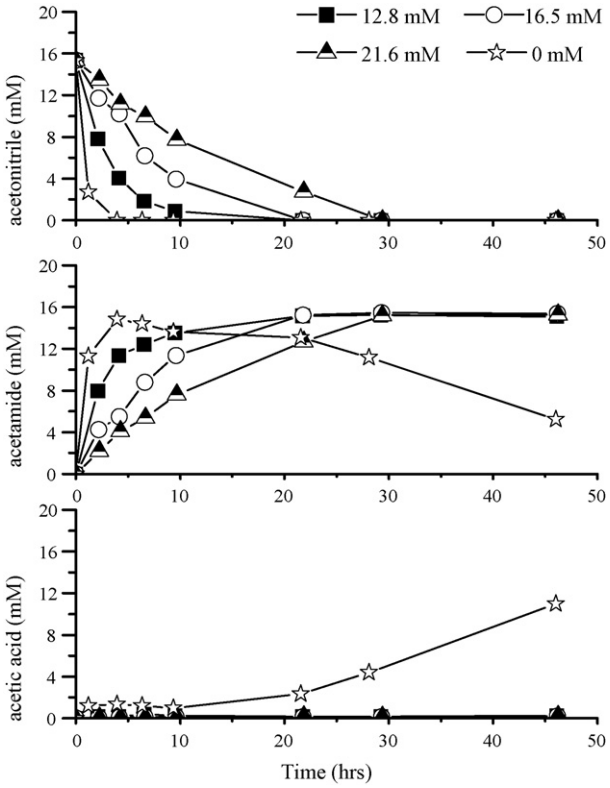

Fig. 4. The conversion of 15.4 mM acetonitrile by *Mesorhizobium* sp. F28 in a batch reactor containing the phosphate buffer medium (pH 7.5) supplementation with 12.8, 16.5, and 21.6 mM acetic acid. The reaction was carried out at 30 °C with 120 rpm shaking.

acetamide, the conversion rates of acetonitrile were 82% at 1.2 h, 76% at 1.9 h, 82% at 2 h, and 80% at 2 h, respectively. Additionally, the accumulative concentrations of acetic acid (4.63, 4.99, and 6.01 mM) and ammonia (6.17, 6.27, and 6.68 mM) were lower than that without addition at 46 h. It revealed that the addition of acetamide slightly affected the acetonitrile degradation by *Mesorhizobium* sp. F28. The pH value did not significantly vary during the reaction period. The $O.D._{600}$ values decreased at the initial stage of the reaction but increased gradually when acetic acid was produced (data not shown). It determined that *Mesorhizobium* sp. F28 degraded acetonitrile even in the medium which had contained the concentration of acetamide up to 20.3 mM.

3.4. The effect of acetic acid on the acetonitrile biodegradation

Various concentrations of acetic acid (12.8, 16.5, and 21.6 mM) were supplemented in the PBM (pH 7.5) to investigate the degradation of 15.4 mM acetonitrile by *Mesorhizobium* sp. F28 (Fig. 4). The higher acetic acid concentration was added, and the lower conversion rate was observed. As the addition concentrations of acetic acid were 0, 12.8, 16.5, and 21.6 mM, the conversion rates of acetonitrile were 82% at 1.2 h, 94% at 9.5 h, 74% at 9.6 h, and 82% at 21.9 h, respectively. The addition of acetic acid also affected the conversion of acetamide into acetic acid. The acetamide continuously accumulated up to 15.3 mM, and traces of acetic acid (0.21–0.25 mM) and ammonia (0.29–0.30 mM) were produced.

When acetic acid was added to the medium, the growth of cell was reduced and the pH value decreased from 7.5 to 4.7–5.5 (data not shown). The possibility of the effect of pH value might be excluded since the low pH value did not depress the conversion of acetonitrile. To further confirm the inhibitory effect that was due

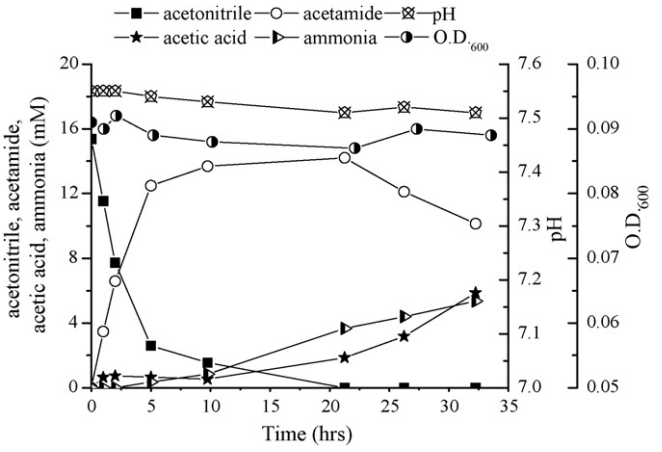

Fig. 5. The conversion of 15.4 mM acetonitrile by *Mesorhizobium* sp. F28 in a batch reactor containing the phosphate buffer medium supplementation with 16.5 mM acetic acid. The pH value was adjusted to 7.5 after acetic acid was added, and the reaction was carried out at 30 °C with 120 rpm shaking.

to pH values or acetic acid, the pH value was adjusted to 7.5 after acetic acid was added. Fig. 5 was the biodegradation of 15.4 mM acetonitrile by *Mesorhizobium* sp. F28 when 16.5 mM acetic acid was supplemented in the PBM which the pH value was adjusted after adding acetic acid. The conversion rates of acetonitrile were 49% at 2.0 h and 90% at 9.8 h, respectively. The accumulative acetamide was reached to 14.2 mM at 21.3 h, and decreased to 10.1 mM at 32.3 h. The concentrations of acetic acid and ammonia at the end of reaction were 5.87 and 5.35 mM, respectively. Comparing with the results described above, it exhibited that the adjustment of the pH values after adding acetic acid was useful to increase the acetonitrile biodegradation of *Mesorhizobium* sp. F28. Nevertheless, the addition of acetic acid still affected the conversion rate of acetonitrile and the accumulation of acetic acid and ammonia. Thus, it determined that acetic acid had the product-inhibitory effect on the acetonitrile biodegradation.

The results about the effects of acetamide and acetic acid on the acetonitrile biodegradation indicated that the product inhibition should be considered. The product inhibitory effects on the conversions of other nitriles had been investigated. The nicotinamide and nicotinic acid which were the products of 3-cyanopyridine biotransformation by *Brevibacterium* R-312 had product-inhibitory effects on the nitrile hydratase [20]. *Bacillus* strain RAPc8 contained a nitrile hydratase-amidase enzyme system that transformed acrylonitrile to acrylic acid. The product-inhibitory results exhibited that there was significant inhibition on the nitrile hydratase activity as acrylic acid concentrations above 200 mM [21]. The research determined that the bioreactor should be properly operated to minimize the deactivation of nitrile hydratase and amidase by high accumulation of acrylamide and ammonium acrylate [22]. The nitrile hydratase activity decreased by product inhibition as *Microbacterium imperiale* CBS 498-74 converted benzonitrile at low temperature [19]. Accordingly, the effects of product inhibition should be noticed as using a nitrile hydratase-amidase enzyme system to convert nitriles.

4. Conclusion

Mesorizobium sp. F28 has the NHase/amidase enzyme system to convert acetonitrile into acetic acid via acetamide as an intermediate. This strain efficiently converted 19.5 mM acetonitrile into acetic acid within 4 h, and used the accumulative acetic acid as a substrate to grow. The pH value could be adjusted between 6.5

and 8.3 to maintain the amidase activity for complete conversion of acetamide into acetic acid. Furthermore, the accumulation of acetamide did not significantly inhibit the conversion of acetonitrile into acetamide by the NHase or the conversion of acetamide into acetic acid by the amidase, but high concentration of acetic acid in the medium affected the biodegradation of acetonitrile by *Mesorizobium* sp. F28. Therefore, the appropriate operation (flow rate, HRT, feed load, and so on) of the bioreactor would be conducted to avoid the accumulation of acetic acid in the reactor as using *Mesorizobium* sp. F28 to continuously degrade acetonitrile.

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