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Establishment of Bioprocess for Synthesis of Nicotinamide by Recombinant *Escherichia coli* Expressing High-Molecular-Mass Nitrile Hydratase

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Abstract Application of engineered bacteria expressing nitrile hydratase for the production of amide is getting tremendous attention due to the rapid development of recombinant DNA technique. This study evaluated the effect of 3-cyanopyridine concentrations on nicotinamide production using recombinant *Escherichia coli* strain (BAG) expressing high-molecular-mass nitrile hydratase from *Rhodococcus rhodochrous* J1, and established proper process of whole-cell catalysis of 3-cyanopyridine and high cell-density cultivation. The process of substrate fed-batch was applied in the production of nicotinamide, and the concentration of product reached 390 g/L under the condition of low cell-density. After the high cell-density cultivation of BAG in 5 L bioreactor, the OD_{600} of cell attained 200 and the total activity reached 2813 U/mL. Different high density of BAG after fermentation in the tank was used to catalyze 3-cyanopyridine, and the concentration of nicotinamide reached to 508 g/L in just 60 min. The productivity of BAG was 212% higher than that of *R. rhodochrous* J1, and it is possible that BAG is able to achieve industrial production of nicotinamide.

Keywords *Escherichia coli* · High-molecular-mass nitrile hydratase · Whole-cell catalysis · Substrate fed-batch · High cell-density cultivation

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Introduction

Nitrile hydratase (NHase, EC 4.2.1.84) [1, 2], which is composed of α- and β- subunits, contains either non-heme iron [3, 4] or non-corrin cobalt ion [5–7] in the activity center. NHase can efficiently catalyze nitrile to the corresponding amide and mainly used for industrial production of acrylamide and nicotinamide [8]. Most of NHases come from *Rhodococcus*, *Pseudonocardia*, and *Nocardia* [9].

As green production is getting more and more attention, the traditional chemical methods have given priority to biological methods consisting of enzyme and whole-cell catalysis [10, 11] and NHase is a classical example of biological production [8]. Due to the high-value product and high catalytic activity, NHase has attracted wide attention in the green industry [12]. Nicotinamide, a kind of important intermediates for drug and pesticide synthesis, also has a wide range of industry applications in the medicinal and food industries [13, 14]. Nowadays, the strain used to produce nicotinamide is *Rhodococcus rhodochrous* J1 (*R. rhodochrous* J1), which is also the third-generation industrial strains producing acrylamide [15, 16]. *R. rhodochrous* J1 produces high- and low-molecular-mass NHases (H-NHase and L-NHase, respectively), which exhibit different physicochemical properties and substrate specificities [1, 17]. Although *R. rhodochrous* J1 was mainly applied in industrial production, it has a few limits such as the long cultivation time and low quantity of protein expression [18, 19]. *Escherichia coli* (*E. coli*) expression system, however, possesses advantages in protein expression and growth rate. Some researchers tried to use recombinant *E. coli* to produce nicotinamide [14]; however, the yield of products was lower than that produced by *R. rhodochrous* J1 [20].

In this study, a recombinant strain *E. coli* BL21 (DE3)/pET-24a (+)-*nhhBrbsArbsG* (BAG) used for H-NHase expression was constructed (unpublished data) and used for producing nicotinamide. Proper bioprocess of catalysis of 3-cyanopyridine and high cell-density cultivation using recombinant *E. coli* were established. Furthermore, the actual industrial catalysis was also simulated to produce nicotinamide. The productivity of BAG was 212% higher than that of *R. rhodochrous* J1, and it is possible that BAG is able to achieve industrial production of nicotinamide.

Materials and Methods

Bacterial Strain and Culture Media in Flask

E. coli BL21(DE3)/pET-24a(+)-*nhhBrbsArbsG* (BAG) was constructed and preserved in our laboratory. The strain was firstly grown in Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin and then the cells were transformed into 2YT medium supplemented with 50 μg/mL kanamycin.

NHase Expression (in Flask) and Cell Activity Assay

E. coli cells carrying the recombinant plasmid were cultured in the 2YT medium with kanamycin at 37 °C. Isopropyl β-D-thiogalactopyranoside (IPTG) and $CoCl_2 \cdot 6H_2O$ were added to final concentration of 0.6 mM and 0.1 g/L to induce protein expression when the OD_{600} reached 0.6–1. The cells were cultured for 18 h at 30 °C for protein expression.

The cell activity was measured in a reaction system (500 μL) consisting of recombinant cells (OD = 0.2), the substrate (100 mM 3-cyanopyridine), and the buffer (10 mM KPB, pH 7.5), which was placed for 10 min at 26 °C and stopped by addition of 500 μL acetonitrile. After the termination of reaction, the tube was centrifuged at 4 °C, 12,000 rpm for 1 min and then the supernatant was extracted for the following detection. The product (nicotinamide) concentration was detected by high-pressure liquid chromatography (HPLC) to measure the BAG's activity. The quantity (μmol) of nicotinamide that 1 mL fermentation liquor produced per minute at 26 °C was defined as units per milliliter.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The expression of the recombinant NHase was analyzed using SDS-PAGE (12%) with a 5% stacking gel. The cells were collected by centrifuge and broken by sonication. The supernatants were mixed with SDS-PAGE loading buffer and loaded onto the gels. All samples were denatured in a boiling water bath for 10 min.

Effect of 3-Cyanopyridine Concentrations on Nicotinamide Production

To study the effect of substrate concentration on production of nicotinamide, the concentration of 3-cyanopyridine (0.2–1.0 mol/L) was varied in a 30-mL reaction mixture containing cells of which OD_{600} value was 8. The reaction's temperature was maintained at 26 °C, and the concentration of 3-cyanopyridine was determined by HPLC.

Fed-Batch Reaction for Nicotinamide Production

According to the effect of 3-cyanopyridine concentrations on nicotinamide, feeding of 41.64 g/L (0.4 mol/L) substrate (powder) was performed interval in a 250-mL flask containing 30 mL reaction mixture with cells of which OD_{600} values were 8.0, 80, 120, and 160. The reaction's temperature was maintained at 26 °C, and a sample was withdrawn before every feed for 3-cyanopyridine.

Fed-Batch Cultivation in 5-L Fermenter Using BAG

Fed-batch cultivations were performed in a 5-L bioreactor with an initial 2 L of medium (12.0 g/L glucose, 13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.7 g citric acid, 1.68 g $MgSO_4$, and 10 mL trace metal solution consisting 10.0 g/L $FeSO_4{\cdot}7H_2O$, 2.0 g/L $CaCl_2$, 5.25 g/L $ZnSO_4{\cdot}7H_2O$, 0.5 g/L $MnSO_4{\cdot}4H_2O$, 3.0 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L $(NH_4)_6MoO_{24}{\cdot}4H_2O$, and 0.23 g/L $Na_2B_4O_7{\cdot}10H_2O$ dissolved in 1 M HCl). The preinoculum culture was 120 mL LB liquid medium supplemented with 50 μg/mL kanamycin and shaken at 200 rpm at 37 °C. After 7.5 h, the culture was inoculated into the fermenter under sterile conditions. The inoculation volume was 6%. The culture was performed at 37 °C for about 6 h until the dissolved oxygen (DO) suddenly rose. Subsequently, the feed medium (500 g/L glucose, 4 g/L yeast extract, 4 g/L tryptone, and 7.33 g/L $MgSO_4$) was added to the fermenter by exponential feeding. The exponential feeding strategy allows cells to grow at a constant specific growth rate by using glucose as a growth-limiting nutrient. When the OD_{600} reached to 60, the temperature was gradually reduced to 30 °C and we added mixed solution containing lactose (10%, *w*/*v*) and $CoCl_2{\cdot}6H_2O$ (9.3%, *w*/*v*) at speed of 0.22 g/(L·h). During the entire process, the pH was maintained at 7.0 through the automatic addition of ammonium solution (25%, *w*/*v*).

Antifoam was added manually when necessary. The dissolved oxygen level (DO) was maintained at approximately 30% air saturation throughout fermentation. The initial airflow rate was 5 L/min, and increased with the need for DO. The end of the cultivation was determined by a reduction in the oxygen consumption rate and an increase in the pH value.

Results and Discussion

Effect of 3-Cyanopyridine Concentrations on Nicotinamide Production

The recombinant strain BAG was incubated for H-NHase expression. The NHase activity reached maximum (95.8 U/mL) after incubation for 18 h, and the OD_{600} of cells reached 8.68 (Fig. 1A). SDS-PAGE analysis indicated that H-NHase was successfully expressed (Fig. 1B). In order to establish proper process of catalysis, the effect of 3-cyanopyridine concentration on the catalytic efficiency of the H-NHase was examined. The recombinant cells after incubation for 18 h were collected and then suspended in 10 mM KPB (OD_{600} = 8); Each of 0.2, 0.4, 0.6, 0.8, and 1.0 mol/L 3-cyanopyridine (final concentration) was mixed with the suspended cells, the reaction rate was calculated after the 3-cyanopyridine was completely converted into nicotinamide. As shown in Fig. 2, when the original concentration of 3-cyanopyridine was 0.4 mol/L, it showed the highest reaction rate. The catalytic rate decreased with increasing 3-cyanopyridine concentration (0.6–0.8 mol/L), suggesting substrate inhibition.

Fed-Batch Reaction for Nicotinamide Production

The method of substrate-flow was originally intended to apply to achieve the hydration reaction, like the production of acrylamide [21]. However, the maximum solubility of 3-

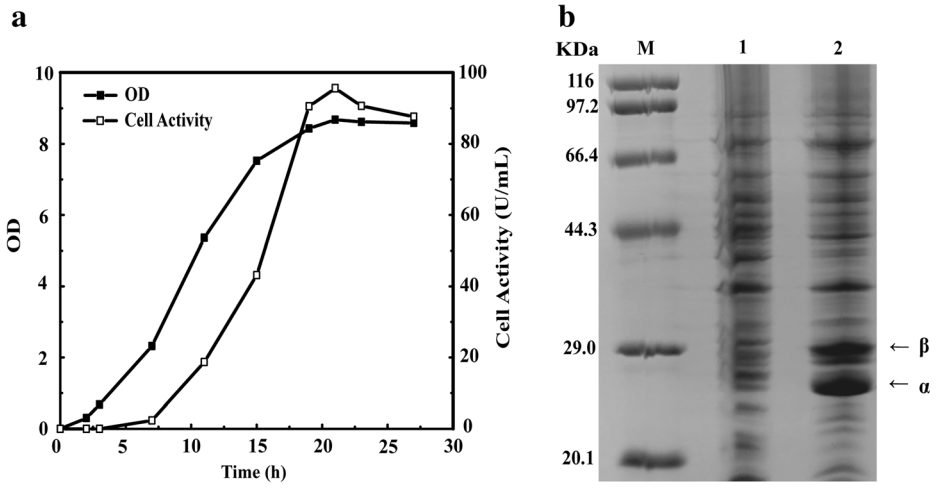

Fig. 1 Batch culture course in 250 mL flask of BAG. **a** Cell growth and NHase activity during batch cultivation. **b** SDS-PAGE analysis of the NHase gene expression in the recombinant strain. The cells were collected after 18 h induction by centrifuge and broken by sonication, and then the supernatants were loaded onto the gels. *M*: protein molecular mass markers; *1*: Supernatants of BL21(DE3)/pET-24a (+) expression as a comparison; *2*: Supernatants of BAG

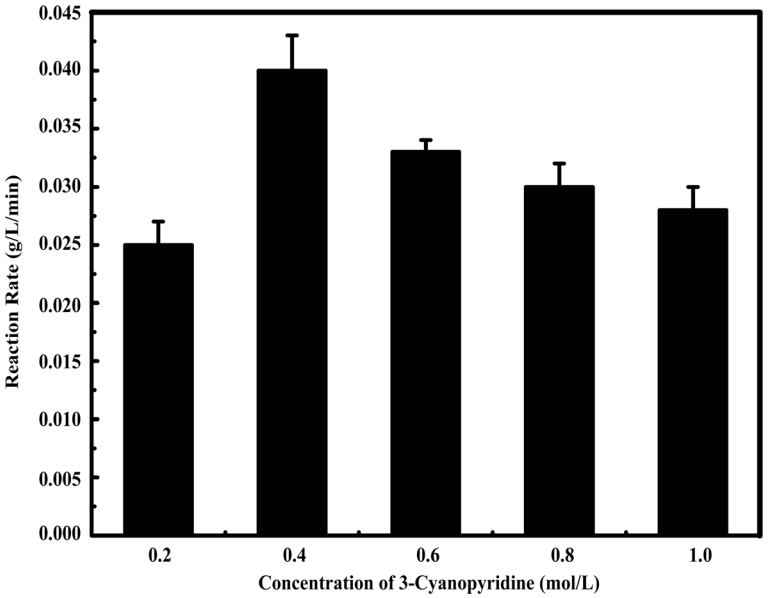

Fig. 2 Effect of 3-cyanopyridine concentration on the catalytic efficiency of the H-NHase. Feeding of different concentration (0.2–1.0 mol/L) of 3-cyanopyridine (powder) was performed interval in a 250-mL flask containing 30 mL reaction mixture with cells of which OD_{600} value were 8.0. The temperature was maintained at 26 °C

cyanopyridine is about 1 mol/L and it failed to obtain high concentration of nicotinamide. Thus, a fed-batch reaction using solid powders of 3-cyanopyridine was carried out to gain our target compound. Solid powders of 3-cyanopyridine were added to the reaction system which contained 30 mL of cells (OD_{600} = 8), and the next batch of substrate were not added until remaining substrate was depleted. To maintain the highest reaction rate, 3-cyanopyridine was added 0.4 mol/L each time. At the beginning of the reaction, it took 10 min to convert the substrate and the catalytic rate could be maintained until the concentration of product reached to nearly 250 g/L (Fig. 3). Then, the reaction rate decreased subsequently and 390 g/L nicotinamide was obtained at 105 min.

Fed-Batch Cultivation of BAG in 5-L Fermenter

The highest OD_{600} of cells after the flask cultivation was about 8, which failed to meet the requirement of industrial production and achieve high reaction rate. Therefore, it is necessary to establish high cell-density cultivation of recombinant *E. coli*, which is also an effective way to enhance the expression of heterologous protein. In this study, fed-batch cultivation was applied to achieve the high-density cultivation of BAG. The time course for a representative cultivation in a 5-L fermenter and other parameters was shown in Fig. 4. As shown in Fig. 4A, the DO reached to 100% sharply after 6 h incubation and the feeding process was started. When the OD_{600} was almost 60, the temperature was gradually reduced to 30 °C to induce the expression of the recombinant NHase. The OD_{600} of cells was about 200 at 44 h, and the highest total activity reached about 2813 U/mL. There was no accumulation of glucose, and the concentration of acetic acid was no more than 0.5 g/L during the fed-batch cultivation. SDS-PAGE analysis showed that the expression level of H-NHase per unit biomass was

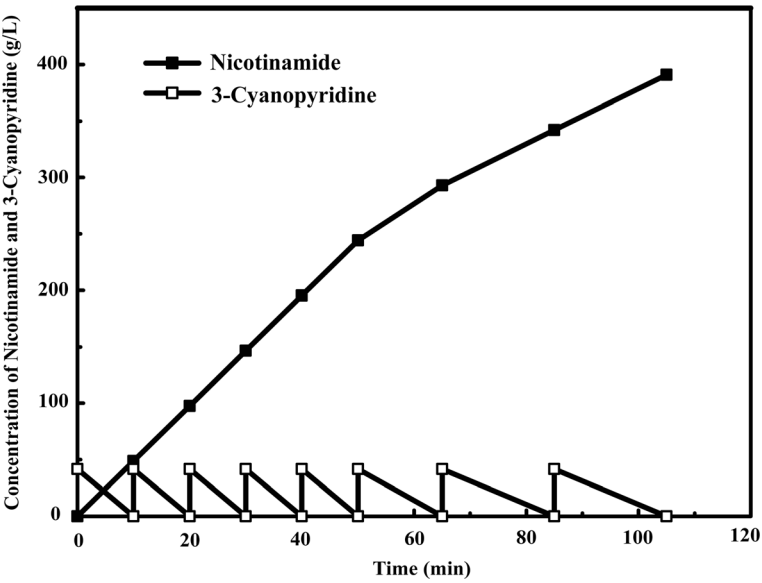

Fig. 3 Hydration reaction of 3-cyanopyridine to nicotinamide catalyzed by BAG during the fed-batch reaction. Feeding of 41.64 g/L (0.4 mol/L) 3-cyanopyridine (powder) was performed interval in a 250-mL flask containing 30 mL reaction mixture with cells of which OD_{600} value were 8.0. The temperature was maintained at 26 °C

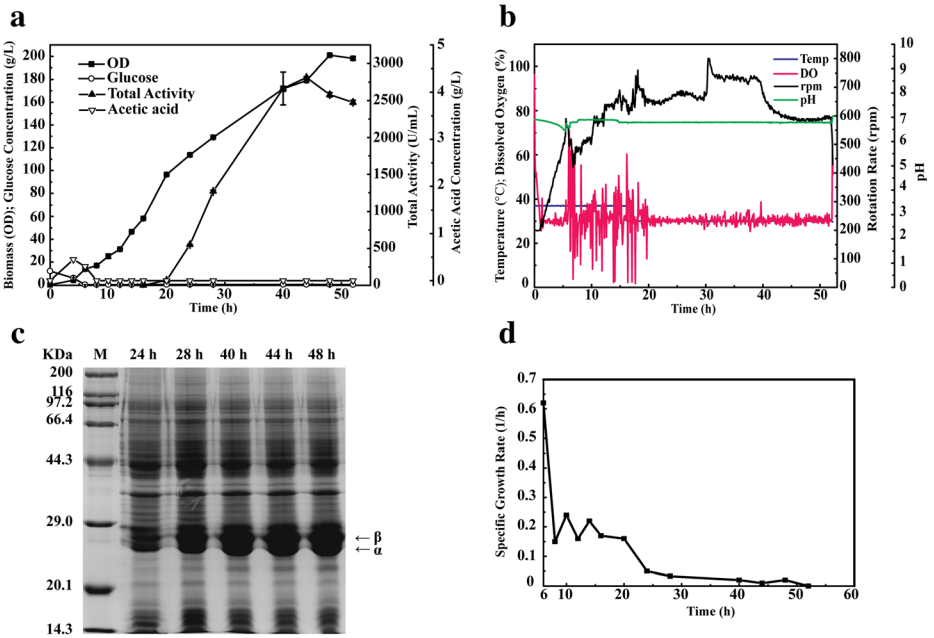

Fig. 4 High-density fermentation of BAG in a 5-L fermenter. **a** Cell growth and NHase activity, concentrations of glucose and acetic acid during fed-batch cultivation. **b** Changes of pH, rotation rate, dissolve oxygen, and temperature during fed-batch cultivation. **c** SDS-PAGE analysis of the NHase gene expression in the recombinant strain. The cells were collected at different time and broken by sonication, and then the supernatants were loaded onto the gels. *M*: protein molecular mass markers; *24–48 h*: protein expression in different time of growth. **d** Specific growth rate of cells during fed-batch cultivation

increased with time (Fig. 4C). The specific growth rate (Fig. 4D) fluctuated around 0.2 h^{-1} during the period when exponential feeding strategy was performed (6–16 h).

Nicotinamide Production by High Density of BAG

In order to be closer to the actual production situation in enterprises, high density of BAG, which was cultivated and induced in 5-L fermenter, was used to catalyze. Three different density of BAG (OD_{600} = 80, 120, 160) was applied to catalyze 3-cyanopyridine. As shown in Fig. 5, at the beginning of the reaction, it took just a few minutes to convert the substrate completely because of the high cell-density. However, when the concentration of nicotinamide reached to about 340 g/L, the catalytic rate started to reduce whatever the OD_{600} were 80, 120, or 160. The higher OD of cells, the slower the rate decreased. The highest concentration of nicotinamide we obtained was 508 g/L at reaction system which OD_{600} of cells was 160 in just 1 h (Fig. 5C). Higher density of cells could improve the original rate of reaction and shorten the catalytic time, but it could not change cells' tolerance to product. It could be seen that final concentration of the product produced by 160 OD_{600} cells is just 6.25% higher than that of the product produced by 120 OD_{600} cells (Fig. 5B). It could also be predicted that when the density of cells was higher than 160 OD_{600}, the final

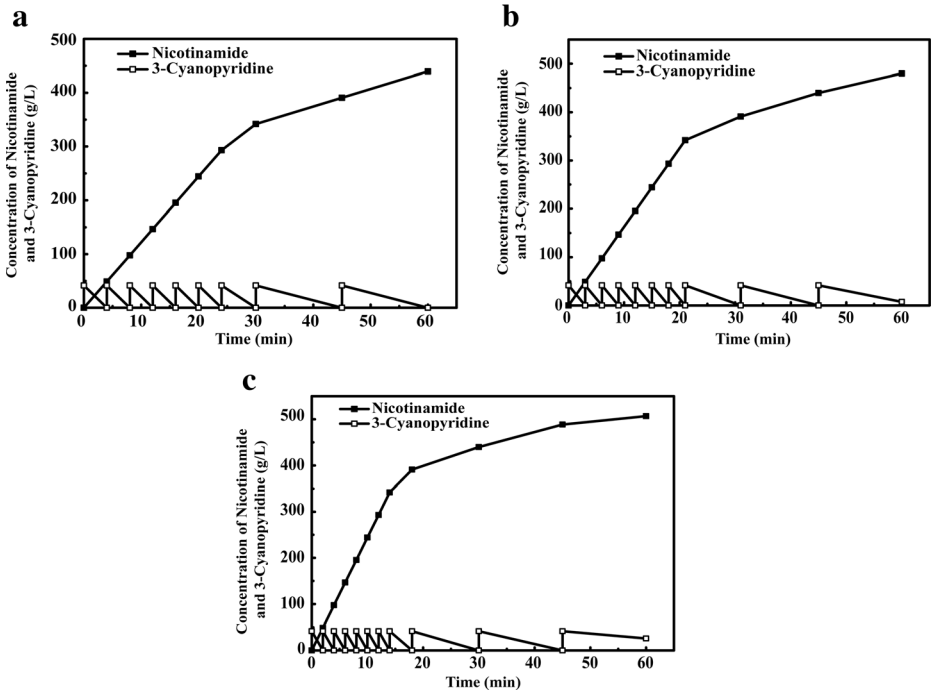

Fig. 5 Nicotinamide production by different high density of BAG. Feeding of 41.64 g/L (0.4 mol/L) 3-cyanopyridine (powder) was performed interval in a 250-mL flask containing 30 mL reaction mixture with BAG which was cultivated and induced in 5-L fermenter. The temperature was maintained at 26 °C. The reactions were terminated at 60 min. **a** OD_{600} of cell was 80. **b** OD_{600} of cell was 120. **c** OD_{600} of cell was 160

concentration of nicotinamide would be more than 508 g/L but not much too higher. Thus, in industrial production, there was no need to choose the highest cell density.

Not like *R. rhodochrous* J1, the tolerance to organic solution of *E. coli* was limited. Nevertheless, the fermentation period of *R. rhodochrous* J1 was up to 100 h [18] and the cell activity (2100 U/mL) [22] was 34% lower than that of BAG. By contrast, BAG's fermentation time was just half of that of *R. rhodochrous* J1 and the product concentration can reach the standard of industry by using shorter catalytic time. The productivity of BAG was 508 g/(L·h), which was 212% higher than that of *R. rhodochrous* J1 (162.8 g/(L·h)) [20]. As mentioned above, *R. rhodochrous* J1 also produces L-NHase, and a recombinant strain *E. coli* containing *L-NHase* genes (BAE) was constructed. Though the BAE possessed higher activity than BAG, during the process of catalysis, cell activity of BAE dropped sharply in the high concentration of production. When the concentration of nicotinamide was 1.0 mol/L, the relative enzyme activity of BAE was no more than 10%, while the BAG still kept its catalytic ability more than 70% at the same concentration of nicotinamide.

The BAG was also used for producing acrylamide, another industrial production used by NHase, and the final concentration of acrylamide reached 44% at reaction system which OD_{600} of cell was 160 in just 1 h. The short fermentation period and high quantity of protein expression will make BAG possess broad application prospect. Of course, extensive researches should be conducted to improve the cell and enzyme's tolerance to nicotinamide and acrylamide in order to gain higher concentration of production.

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