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Efficient Separation of Cytosine-Substituted Mildiomycin Analogue (MIL-C) from the Fermentation Broth by Ion Exchange

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Abstract: Cytosine-substituted mildiomycin analogue (MIL-C) was synthesized by supplementing cytosine into the culture medium of *Streptoverticillium rimofaciens* (one mildiomycin producer) and had a specific and strong inhibitory activity against powdery mildews. In order to facilitate the separation of MIL-C from this fermentation broth, the cultivation conditions were optimized to increase MIL-C productivity, and more than 0.9 g/l cytosine was added to the medium to inhibit the biosynthesis of mildiomycin (MIL). According to the ion-exchange equilibrium and dynamics characteristic of MIL-C, one weakly cationic resin (DK110) was screened out to separate MIL-C from fermentation broth with one effective ion-exchange method. When 2% ammonia aqueous solution was applied as eluent with 2 BVs/h, high recovery yield (97.6%) and purity (70.0%) of MIL-C were achieved. This novel strategy of combining up-stream biosynthetic controls and down-stream purification is very promising for efficient production of this novel nucleoside antibiotic (MIL-C).

Keywords: Adsorption, desorption, mildiomycin cytosine analogue, weakly cationic exchange resin

INTRODUCTION

Mildiomycin (MIL) is a novel nucleoside agro-antibiotic with a specific and strong inhibitory activity against powdery mildews. MIL is regarded as a

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kind of green biological pesticide, because of its low action dosage, excellent environmental compatibility, and remarkably low toxicity to human and animals (1–3). It has been found that a variety of new mildiomycin analogues can be ingathered when the culture medium is supplemented with an analogue of 5-hydroxymethyl cytosine, such as cytosine, 5-fluorin cytosine and 5-bromine cytosine etc. which is one of the moieties in mildiomycin molecule (4).

According to the preliminary study in this laboratory, the bioactivities of cytosine-substituted mildiomycin analogue (MIL-C) for controlling powdery mildew of plants in greenhouse and field tests were much higher than that of MIL, and the toxicity was much lower (5). The comparison of the molecular structure of MIL and MIL-C is shown in Fig. 1. In each MIL molecule, there are four ionized groups that can be dissociated: one amino group in serine residue, another amino group in cytosine moiety, one carboxyl group, and one carbamidine group, and their pK values are 4.2, 7.2, 2.8, and >12, respectively. The isoelectric points of MIL is about pH 10.0, which means that MIL will be positively charged when the pH value is below 10.0, therefore, it has proved to be feasible to separate MIL from fermentation broth by ion exchange (3, 6). The molecular structure of MIL-C is similar to MIL with the only difference in cytosine moiety. They have the same four dissociable groups and isoelectric points. Therefore, it is possible to employ the ion-exchange method to separate this new mildiomycin analogue (MIL-C) from the fermentation broth.

Although some literatures were reported to employ the ion-exchange chromatography to separate MIL from the fermentation broth, few efforts were paid to separate its analogue from the co-existing MIL fermentation broth (3, 6). In our previous work, one weakly acid cationic exchange resin (HZ011 resin) was developed to separate MIL efficiently, but can not be used to separate MIL and MIL-C effectively because of its high adsorption capacity for both of them. It was found in this laboratory that MIL and MIL-C could be synthesized concomitantly in the broth when a certain quantity of cytosine was supplemented to the fermentation medium with

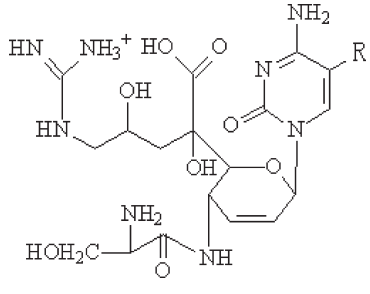

Figure 1. Molecular structure of MIL and MIL-C (MIL: R = CH_2OH; MIL-C: R = H).

Streptoverticillium rimofaciens. Apparently, the simultaneous biosyntheses of MIL and MIL-C will put additional difficulty to separate MIL-C from this fermentation broth because of their similar physical and chemical properties. To solve this separation problem, we first focused on the investigation of the possibility of inhibiting the biosynthesis of MIL by adjusting culture conditions, then contributed to the development of one ion-exchange method to separate MIL-C from the broth effectively. The results showed that the biosynthesis of MIL could be inhibited severely by adding high concentration of cytosine into the medium, and one effective ion-exchange procedure was developed to separate MIL-C with high recovery rate and product purity. This novel strategy of combining up-stream biosynthetic controls and down-stream purification is very encouraging for the production of MIL-C, and will be also helpful for the development of an other similar analogue separation process.

MATERIALS AND METHODS

Strains and Media

Streptoverticillium rimofaciens ZJU 5119 was maintained in China General Microbiological Culture Collection (CGMCC, with accession number CGMCC 1503).

The media used for the slant, seed and fermentation cultures were as follows. Slant medium contains (g/l) soluble starch 20, KNO_3 1.0, NaCl 0.5, $K_2HPO_4 \cdot 3H_2O$ 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, Peptone 5.0, and Agar 20. The seed medium contains (g/l) glucose 20, soybean cake meal 30, yeast extraction 5.0, $(NH_4)_2\ SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCO_3$ 3.0. Fermentation medium (g/l): glucose 18.7, rice mill 64.8, peanut cake powder 65.12, KNO_3 0.50, K_2HPO_4 0.45, NaCl 0.25, $MgSO_4$ 0.35, $FeSO_4$ 0.015, N, N-Dimethylacetamine 1.13, $CaCO_3$ 10.0.

Materials

One weak acid cationic exchange resin (DK110) was purchased from Shanghai Huazhen Resin Corporation, Shanghai, China. The main characteristics of the DK110 are summarized in Table 1. All chemicals are in AR grade or better.

Pretreatment of Resin

The resin was pre-treated by repeated washes with 1 M HCl and 1 M NaOH solutions, and then converted to the hydrogen form by elution with 2 M HCl and rinsed at neutral pH with deionized water.

Table 1. Characteristics of DK110 cationic exchange resin

Matrix	Acrylic acid
Functional group	Carboxyl
Ionic form	H^+
Exchange capacity by volume	1.75 mM
Exchange capacity by quality	>8.0 mmol/g of dry mass
Effective size	0.3–1.2 mm
Regeneration	1 M NaOH, 1 M HCl

MIL-C Production with *Str. rimofaciens* ZJU 5119

For the production of MIL-C, all the fermentations were inoculated with Str. rimofaciens ZJU 5119 and cultivated at 28°C in the 500-ml shake-flask containing 80 ml fermentation medium. Different concentration of cytosine (from 0 g/l to 1.2 g/l) was added to the medium to investigate the effect of cytosine on the production of MIL-C after 8-day cultivation.

Separation of MIL-C from Fermentation Broth

After an 8-day cultivation, the fermentation broth was harvested and the pH value was adjusted to 3.0, and then was centrifuged at 4000×g for 10 min to remove the mycelia. Then the pH value of the supernatant was readjusted to 7.5 for the determination of MIL-C.

The ion-exchange equilibrium of MIL-C on DK 110 resin was studied in a shaker at 200 rpm with temperature control. Adding 50 ml of the supernatant with known MIL-C concentration and 1.0 g of wet resin into a 250 ml shake-flask located in the shaker, the ion exchange amount (q, mg/g resin) of MIL-C onto DK 110 resin was calculated from the difference between initial (C_0, mg/l) and final (C_t, mg/l) MIL-C concentrations.

$$q = \frac{(C_0 - C_t) \times v}{W} \tag{1}$$

where, v is the volume of liquid phase (l) and W is the weight of resin (g).

For the purpose of determining ion-exchange dynamics of MIL-C on DK 110 resin, 0.5 ml of liquid sample was taken to analyze the MIL-C concentration in certain time interval.

An ion-exchange column (124 × 10 mm) was set-up and filled with 7 ml of wet DK110 resin. The column was pre-treated by repeated washes with 1 M HCl, 1 M NaOH solutions and then washed with a large amount of deionized water. Then the fermentation broth at pH 7.0 was pumped into the column for ion exchange. The adsorption step was stopped after the concentration of MIL-C in the effluent is closed to the initial broth. The column was washed first by deionized water with high flow rate (over 10 BVs/h, bed volume

per hour) to elute most of cytosine, which could not be adsorbed by the DK110 resin at pH 7.0. The desorption process of MIL-C were then optimized with different eluent and different flow rate.

Analytical Methods

An Agilent 1100 series HPLC System equipped with a Hypersil BDS C_{18} column (250 × 4.6 mm) was employed to analyze the MIL-C concentration (7). The column temperature was kept at 25°C. The mobile phase consists of methanol/trichloroacetic acid (1.0%, w/v)/H_2O (80/100/820, by vol.), and the flow-rate is 1.0 ml/min. The wavelength of UV detector was set at 279 nm and the volume of each injection was 10 μl.

A standard curve between the peak area and concentration of MIL and MIL-C was determined by analytical HPLC. The standards (MIL and MIL-C) were prepared from the commercial MIL product and the fermentation broth by using preparative HPLC in the laboratory, respectively. The purity of MIL-C product after elution was determined by drying the elution under vacuum pressure and solving the dried sample in the distilled water, which was analyzed by HPLC based on the standard product.

RESULTS AND DISCUSSIONS

Production of High MIL-C-containing Fermentation Broth

The natural product of *Str. rimofaciens* ZJU 5119 cultivation is MIL; however, the addition of cytosine into the culture medium will cause the product transfer from MIL to MIL-C. As shown in Fig. 2, the addition of cytosine ranged from 0 to 0.3 g/l, the cytosine was completely transferred for the biosynthesis of MIL-C, and the MIL yield was reduced seriously. Further increase of cytosine addition caused the accumulation of cytosine in the fermentation broth and led to the decrease of MIL/MIL-C ratio. When 0.6 g/l cytosine was added to the medium, the highest MIL-C productivity and a very low MIL concentration was obtained in the broth. More than 0.6 g/l cytosine addition would lead to linear increase of residue cytosine concentration in the broth, and the MIL-C productivity could not be further improved.

One interesting result was found in Fig. 2 that no MIL accumulation was observed when more than 0.9 g/l cytosine was added into the fermentation medium. It seems that the biosynthesis of mildiomycin was totally suppressed by such high concentration of cytosine. Considering the difficulty of separate MIL-C from the culture broth containing MIL, 0.9 g/l cytosine addition would fascinate the down-stream purification of MIL-C greatly. According to the molecular structure of mildiomycin (Fig. 1), one biosynthetic pathway of mildiomycin was proposed in *Str. rimofaciens* (8), indicating

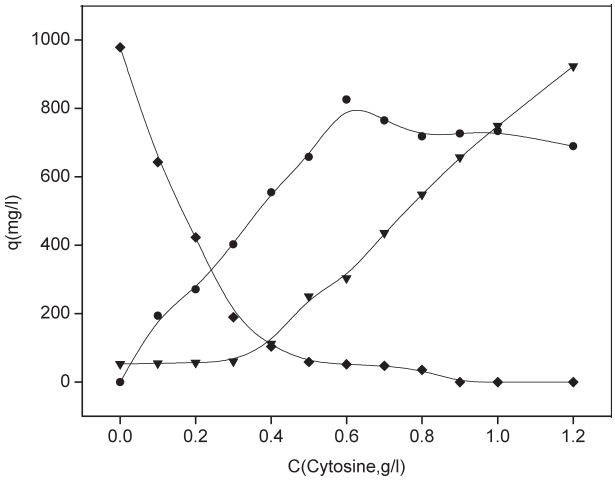

Figure 2. The effect of cytosine addition on the production of MIL-C. (▼): Cytosine, (◆): MIL, (●): MIL-C.

that 5-methylhydroxyl cytosine is employed as one precursor unit for mildiomycin biosynthesis. However, the key enzyme seemed to have relatively broader substance suitability and could utilize cytosine to synthesize mildiomycin analogue instead of 5-methylhydroxyl cytosine. Because of high concentration of available cytosine, this enzymatic reaction will transfer to the biosynthesis of MIL-C. The higher the cytosine concentration is, the less the MIL/MIL-C ratio will be, until MIL was not detected anymore in the fermentation broth. Thus the excess addition of cytosine is necessary to obtain MIL-C as the only product, which is beneficial for the separation and purification of MIL-C from the fermentation broth. The highest MIL-C yield in the batch fermentation was 825 mg/l, and 0.9 g/l cytosine addition was adopted for the preparation of MIL-C broth without MIL.

Ion Exchange Isotherms of MIL-C on DK 110 Resin

Experimental data of ion-exchange isotherms of MIL-C on DK 110 resin at three different temperatures were shown in Fig. 3. Obviously, the ion-exchange capability of MIL-C on DK 110 increases slightly with raising the temperature (9, 10), which indicates that the ion exchange is an endothermal process. The isotherms can be correlated by Langmuir equation (11–13)

$$\frac{1}{q_e} = \frac{1}{bq_0C_e} + \frac{1}{q_0} \tag{2}$$

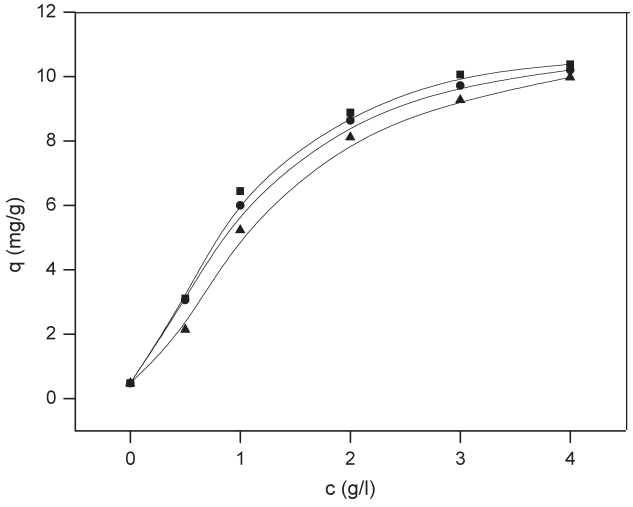

Figure 3. Effect of temperature on MIL-C adsorption on DK110 resin (C_0: 630 mg/l, pH: 7.0) (▲): 293.15 K, (●): 303.15 K, (■): 313.15 K.

A dimensionless constant separation factor (R_L) has been defined to assess the validity of the Langmuir-type adsorption process:

$$R_L = \frac{1}{1 + bC_0} \tag{3}$$

where b is the Langmuir constant and C_0 is the initial concentration of MIL-C in broth (mg/l). The value of R_L indicates the characteristic of the isotherm, which is irreversible ($R_L = 0$), favorable ($0 < R_L < 1$), linear ($R_L = 1$) or unfavorable ($R_L > 1$) (14).

The model parameters were listed in Table 2. The comparisons between experimental data and model simulation were also shown in Fig. 3. The obtained R_L values at three different temperatures show that the adsorption of MIL-C on DK110 is favorable, irrespective of the initial MIL-C concentration. Therefore, high exchange-capacity of MIL-C on this resin can be

Table 2. Parameters of Langmuir isotherm equation of MIL-C on DK 110 resin

T (K)	$1/bq_0$	$1/q_0$	R_L
293.15 K	0.211	0.026	0.0126
303.15 K	0.131	0.055	0.0038
313.15 K	0.129	0.051	0.0039

obtained even if the MIL-C concentration in the broth is very low, which will be very beneficial to separate low MIL-C concentration from the broth.

Ion-exchange Dynamic Property of MIL-C on DK110 Resin

Generally, the ion-exchange reaction is very fast and the major factor affecting the overall ion-exchange rate is the diffusion. The entire rate of the ion-exchange is dependent on both mass transfer resistance and mass-action mechanism. The mass transfer resistance occurred in the ion-exchange process consists of both liquid-film diffusion and intra-particle diffusion. It is important to know which step is the rate-limiting step for the purposes of process enhancement and design (15).

According to Fick's law, for liquid-film diffusion control, the diffusion rate equation can be described as follows (16, 17):

$$\ln(1 - F) = -k_l t \tag{4}$$

where, k_l is the mass transfer rate constant of liquid-film diffusion, $F = q/q_\infty$, q_∞ means the ratio of real amount of ion exchanged over that of equilibrium. It is obvious that a linear relationship will be obtained between $-\ln(1 - F)$ and t in liquid-film diffusion, with the slope of k_l.

As for intra-particle diffusion control, referring to the Fick's law of spherical particles, the uptake rate can be calculated according to the following equation (16):

$$F = 1 - \frac{6}{\pi^2}\sum_{N=1}^{\infty}\frac{1}{n^2}\exp(-n^2 Bt) \tag{5}$$

where, B is the internal diffusion constant, $\mathrm{B} = \mathrm{D}\pi^2/\mathrm{r}_0^2$, and D is the intra-particle diffusivity. For each q value, which can be can be obtained by experiment, a value of Bt is calculated from Eq. (4) Thus, in case of intra-particle diffusion control, Bt should follow a linear relationship to t with the slope of B.

The experimental ion-exchange uptake curve of MIL-C on DK 110 resin was shown in Fig. 4. Only the first four experiment points were true of the ion-exchange dynamic principle of DK110 adsorption because the adsorption reached the equilibrium within 30 min. The relationships between $-\ln(1 - F)$ and t as well as between Bt and t within 30 min were explained in Fig. 5. It is clear that the relationship between $-\ln(1-F)$ and t did not follow the linear whereas the relationship between Bt and t did follow the linear one. The result indicated that the ion-exchange rate of MIL-C on DK 110 resin was controlled by the intra-particle diffusion.

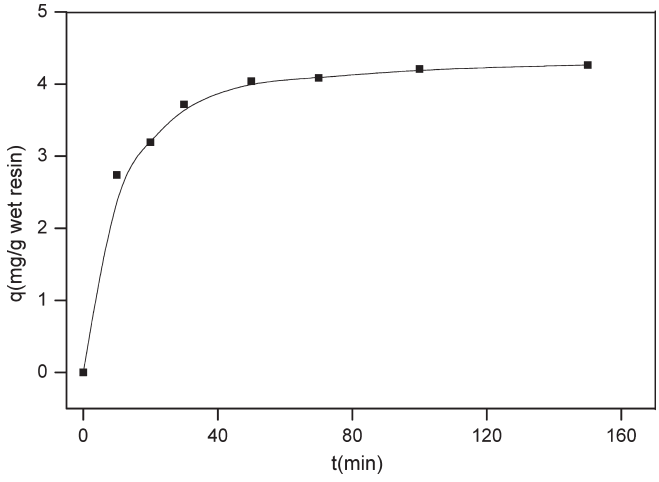

Figure 4. Ion-exchange dynamic curve of MIL-C on DK110 resin (C_0: 630 mg/l, T: 293.15 K, pH: 7.0).

Separation of MIL-C from Fermentation Broth by Ion-exchange Column

Because of no MIL accumulation in the broth, the efforts were paid to separate MIL-C from the residual cytosine and other impurities in the fermentation

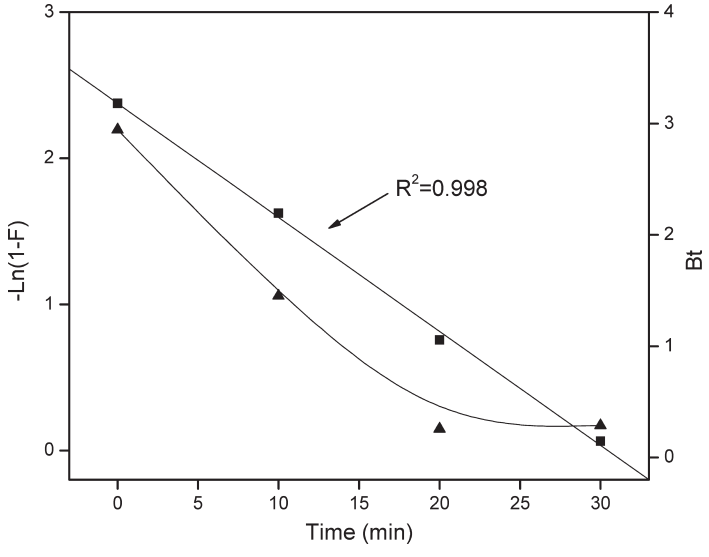

Figure 5. Estimate of interaction mechanism between MIL-C and DK110 resin. (■): Bt, (▲): $-\ln(1-F)$.

broth. When the pH value of the fermentation broth was adjusted at pH 7.5, MIL-C was positively charged because its isoelectric point is 10.0, whereas the isoelectric point of cytosine is 7.5, therefore, it existed in negative charged and molecular forms. Thus, when the fermentation broth passed through a column filled with DK 110 resin, MIL-C was withheld by ion exchange and most of the cytosine was removed. When some remaining cytosine in the void space of the column was washed out with deionized water (pH 6.5), the ion-exchanged MIL-C in the column was ready for elution.

Eluent Selection

Three kinds of desorption solution (2% $NH_3 \cdot H_2O$, 2M NaCl and 1M NH_4Cl) were applied as the eluent to evaluate their ability of releasing MIL-C from DK110. As shown in Fig. 6, all three kinds of eluent are able to desorb MIL-C from the resin and the integral area of each elution curve is at the same level, when the same flow rate (2 BVs/h) is set for each eluent. Obviously, the elution curve of MIL-C with 2% ammonia is the sharpest among three curves. Because of the high isoelectric point of MIL-C (about pH 10.0), the high pH value of 2% ammonia would lead to the sharp elution peak. The sharper the elution peak is, the higher the enrichment factor will be. Another advantage of using ammonia as eluent is the easy removal because of its high volatility, which is very important in the further purification of MIL-C. The MIL-C recovery with ammonia elution was 97.6%. In view of the total recovery ratio, enrich factor and easy removal, 2% ammonia solution was selected as eluent in the following study.

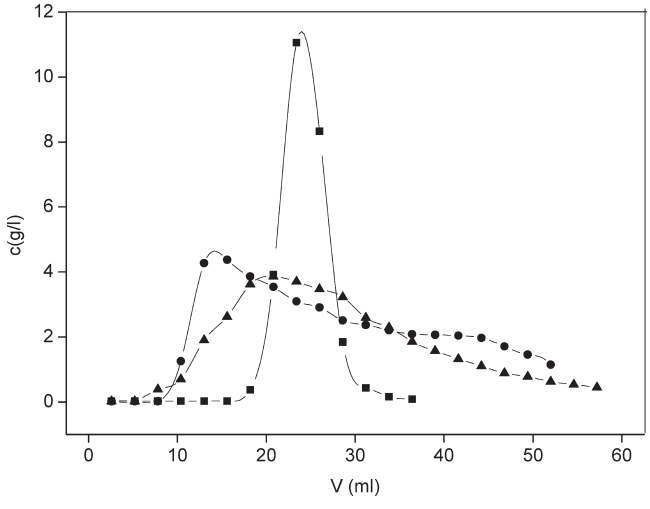

Figure 6. Elution curves with different solutions (T: 293.15 K, L: 124 mm, D: 10 mm). (■): 2% ammonia solution, (●): 2 M NaCl, (▲): 1 M NH_4Cl.

Effect of Eluent Flow Rate on the MIL-C Desorption

Different flow rate of 2% ammonia (1 BV/h, 2 BVs/h and 3 BVs/h) were adopted to desorb MIL-C from DK 110 Resin. The results were shown in Fig. 7. From the uptake curves studied above, it was concluded that the ion exchange rate of MIL-C was controlled by the intra-particle diffusion. Thus, it is reasonable that the low elution rate is also favorable for the desorption of MIL-C from DK 110 resin. With the increase in the flow rate of 2% ammonia solution from 2 BVs/h to 3 BVs/h, the recovery of MIL-C decreased from 97.6% to 86.2%. But the MIL-C is unstable in alkaline solution, the slow flow rate, thus, longer contact time will cause more degradation of MIL-C, therefore, when the flow rate was lowered to 1 BV/h, the MIL-C recovery decreased to 91.6%.

Effect of Ammonia Concentration of Eluent on MIL-C Desorption

Effects of ammonia concentration of eluent on MIL-C desorption from DK 110 resin were studied when the flow rate was kept at 2 BVs/h and the results were shown in Fig. 8. The function of NH_4^+ is to replace the MIL-C adsorbed on the DK 110 resin. When the ammonia concentration was 1% in the eluent, longer time was needed to elute MIL-C and the elution peak was more diffuse. When the NH_4^+ concentration in eluent increased to 2%, more NH_4^+ was available to replace MIL-C, therefore, the MIL-C was purged out of the column faster and the peak became sharper. Further increase of NH_4^+

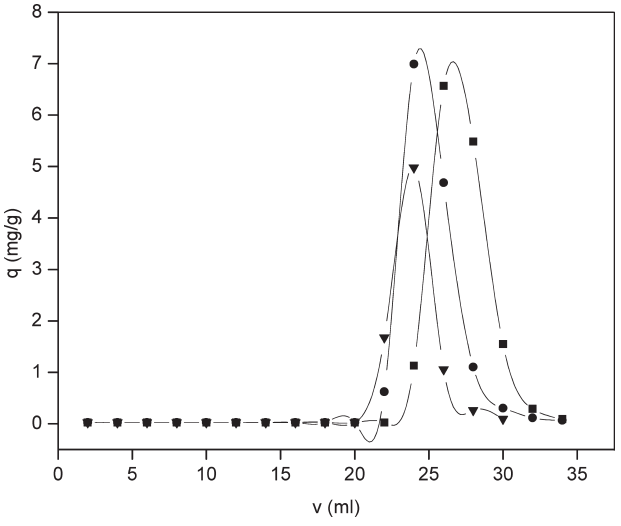

Figure 7. Effect of ammonia flow rate on elution process (T: 293.15 K, L: 124 mm, D: 10 mm). (■): 1 BV/h, (●): 2 BVs/h, (▼): 3 BVs/h.

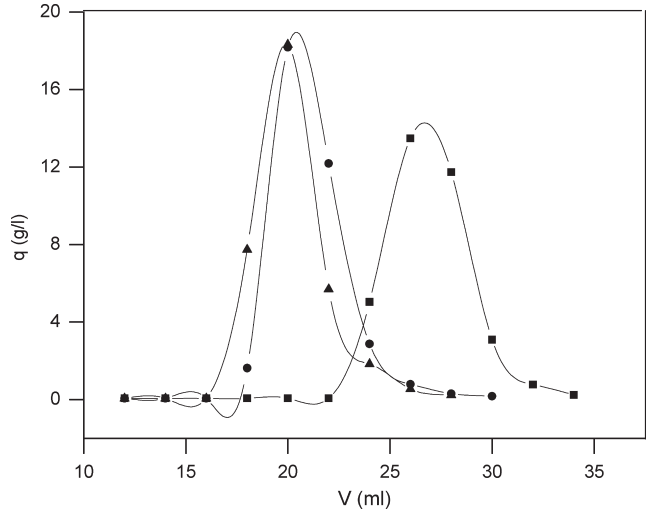

Figure 8. Effect of ammonia concentration on elution process (T: 293.15 K, L: 124 mm, D: 10 mm). (■): 1% ammonia solution, (●): 2% ammonia solution, (▲): 4% ammonia solution.

concentration to 4% caused the excess strengthening of alkalinity, which was harmful for MIL-C stability and resulted in the lowering of MIL-C recovery.

From the above-experimental data, it can be concluded that the favorable eluent is 2% ammonia solution and the suitable flow rate is 2 BVs/h. The

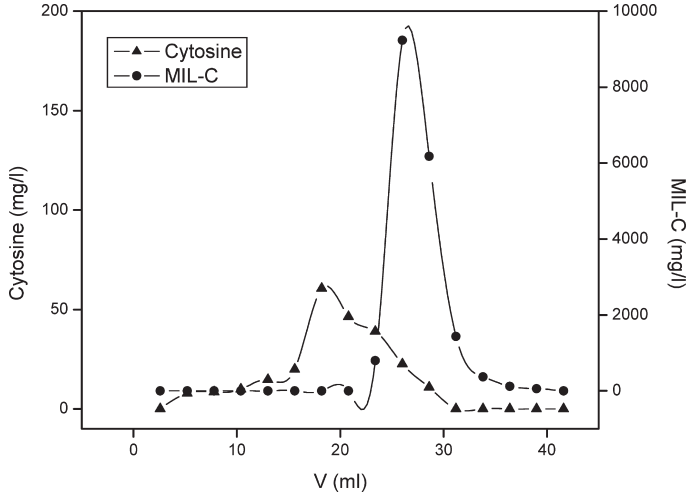

Figure 9. The elution curve of cytosine and MIL-C (T = 293.15 K, 2% ammonia with 2 BVs as eluent, L: 124 mm, D: 10 mm). (▲): Cytosine, (●): MIL-C.

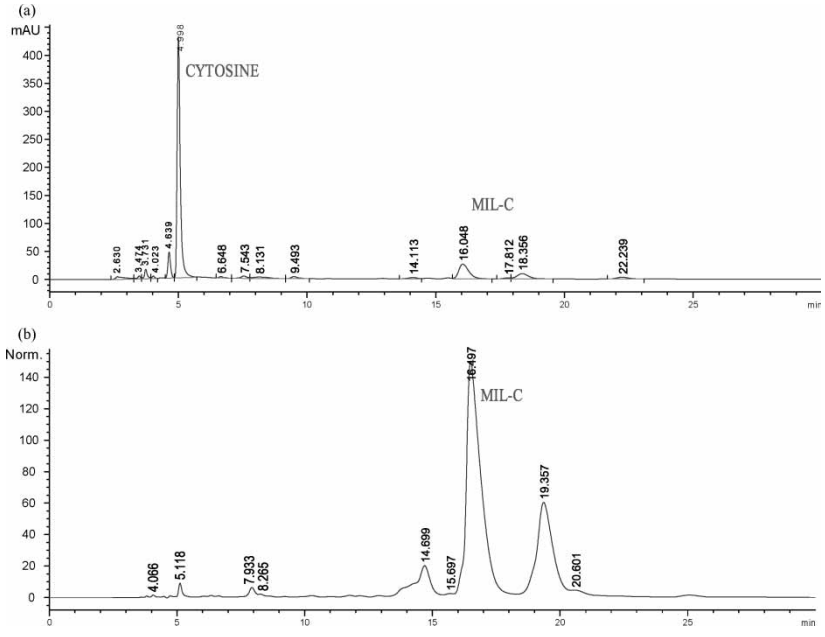

Figure 10. Comparison of HPLC diagrams from the broth supernatant and the elution sample (a: broth supernatant; b: elution sample).

recovery of MIL-C can reach as high as 97.6%. The elution curve of cytosine and MIL-C in such condition was shown in Fig. 9. Under the above separation conditions a relatively high purity 70.0% of MIL-C elution sample was determined by using its vacuum-concentrated sample. The comparison of HPLC chromatograms from the broth supernatant and the elution was carried out in Fig. 10. Apparently, almost all the cytosine was removed by this ionic exchange method, and the concentration of residual cytosine in the eluent is two orders of magnitude smaller than that of MIL-C. Because the commercial mildiomycin product only contained 5.5% MIL, the obtained MIL product with 70.0% purity might be directly formulated to one commercial agrochemical product without further purification. Of course, further purification of this MIL-C product should be studied for a variety of other purpose.

CONCLUSION

The separation procedure of MIL-C was simplified when 0.9 g/l cytosine was added into the culture. In such broth, MIL was inhibited and the main components existed in the broth were MIL-C and cytosine. The cytosine and MIL-C is easy to be separated because of their great difference in structure and characteristics.

By excess addition of cytosine, the MIL biosynthesis was suppressed and MIL-C was the only product during *Str. rimofaciens* ZJU 5119 cultivation. And the separation condition of MIL-C from the fermentation broth by DK 110, a weak cationic exchange resin, was investigated in detail in this paper. The ion-exchange isotherms of MIL-C on DK 110 was measured and correlated by Langmuir equation. The uptake curves of ion exchange of MIL-C on DK 110 showed that the exchange rate was intra-particle diffusion controlled. An ion-exchange column, filled with DK 110 resin, was set-up for MIL-C separation. By adjusting the pH value of the fermentation broth to pH 7.5, the efficient separation between MIL-C and cytosine was achieved. The elution conditions for MIL-C desorption from DK resin were optimized. By using 2% ammonia solution as eluent and a flow rate of 2 BV/h, the recovery of MIL-C reached 97.6% with a relatively high product purity of 70.0%.

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