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Full Length Research Paper

Study on optimal production of 3-ketovalidoxylamine A C-N lyase and glucoside 3-dehydrogenase by a newly isolated *Stenotrophomonas maltrophilia*

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3-Ketovalidoxylamine A C-N lyase and glucoside 3-dehydrogenase (G3DH), two key enzymes for valienamine synthesis, are produced by *Stenotrophomonas maltrophilia*. The condition of producing 3-ketovalidoxylamine A C-N lyase and G3DH was optimized. Validamycin A was showed to be suitable carbon source for C-N lyase and G3DH synthesis. Maximum C-N lyase production was achieved by adding 100 ml culture medium into a 500 ml conical flask containing 5 mg/ml validamycin A, with 12% inoculation seed volume for 22 h with a productivity of 6.26 × 10^{-3} U/mg protein. The specific G3DH activity was 0.244 U/mg protein in the same culture condition at 27 h. A 15 L fermenter containing 8 liter of medium was also used in this study. Crude enzyme reaction showed the conversion of N-*p*-nitrophenylvalidamine was achieved to 92.8% at 3 h and the yield of *p*-nitroaniline was 81.3% at 3 h.

Key words: 3-Ketovalidoxylamine A C-N lyase, glucoside 3-dehydrogenase, validamycin A, valienamine.

INTRODUCTION

It was reported that *Flavobacterium saccharophilum* can use validamycin A as its sole carbon source and degrade validamycin A to validamine, valienamine and unsaturated ketocyclitols by glucoside 3-dehydrogenase (G3DH) and 3-ketovalidoxylamine A C-N lyase (Asano et al., 1984). Another strain producing both G3DH and 3-ketovalidoxylamine A C-N lyase is *Stenotrophomonas maltrophilia*, isolated previously by our laboratory. Valienamine and validamine are strong glucosidase inhibitors (Kameda et al., 1980; Kameda et al., 1984; Chen et al., 2003; Zheng et al., 2005), which could be used as new drugs for diabetes. With the number of people with diabetes increasing, these chemicals are receiving considerable attention. G3DH and 3-ketovalidoxylamine A C-N lyase is greatly important to enzymatic production of valienamine.

In our previous experiments, *S. maltrophilia* was isolated; it could produce G3DH and 3-ketovalidoxylamine A C-N lyase are greatly important to enzymatic production of valienamine (Wang et al., 2007; Zhang et al., 2006). Purification and characterization of G3DH from *S. maltro-philia* has been studied (Zhang et al., 2006). Moreover, the substrate of 3-ketovalidoxylamine A C-N lyase, N-p-nitrophenyl-3-ketovalidamine, was prepared with free cells of *S. maltrophilia* (Zhang et al., 2007).

Some investigations of 3-keto-validoxylamine A C-N lyase and G3DH from *F. saccharophilum* have been reported (Takeuchi et al., 1985; Takeuchi et al., 1986; Takeuchi et al., 1990a; Takeuchi et al., 1990b); the organism was grown in a nutrient medium for enzyme production. However, no other reports on these two enzymes production by *S. maltrophilia* have been published, C-N lyase and G3DH activity from *S. maltrophilia* were rather low using this medium in our previous work (Wang et al., 2007). In order to improve the productivity of valienamine, the conditions of producing these two enzymes should be optimized. In this article, we described the investigation of the conditions for the efficient production of validoxylamine A C-N lyase and G3DH using *S. maltrophilia*.

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Table 1. Effect of carbon source on production of 3-keto-validoxylamine A C-N lyase and G3DH

Carbon source (5 mg/ml)	protein (mg/ml)	G3DH activity (10^{-2} U/mg protein)	Lyase activity (10^{-3} U/mg protein)
Control	0.211	3.39	1.71
Maltose	0.245	5.14	1.80
Sucrose	0.167	5.33	2.96
Lactose	0.209	6.64	3.38
Validamycin A	0.198	23.7	7.05
Acarbose	0.221	2.46	0.64
Glucose	0.207	1.69	0.15
α-methyl-D-glucoside	0.211	9.31	3.42

MATERIALS AND METHODS

Organism and growth

S. maltrophilia was previously isolated from wheat soil near Hangzhou, China, by our laboratory and preserved at the China Center for Type Culture Collection (CCTCC M 204024, Wuhan, China). Minimal culture medium was contained (per liter) 10 g validamycin A, 10 g $(NH_4)_2SO_4$, 5 g NaCl, 2 g K_2HPO_4 and 0.2 g $MgSO_4$. Seed medium was contained (per litre) 5 g beef extract, 10 g peptone, 5 g NaCl and 5 g K_2HPO_4. Batch medium was contained (per litre) 5 g beef extract, 10 g peptone, 5 g NaCl, 5 g K_2HPO_4 and 5 g various carbon sources. The microorganism *S. maltrophilia* was cultured on the above mentioned mineral agar slant for 24 h at 30°C and was then inoculated into a 500 ml conical flask containing 100 ml of sterilized seed medium and cultured at 30°C for 24 h on an orbital shaker of 150 rpm. Batch culture was carried out at 30°C into a 500 ml conical flask containing 100 ml of sterilized batch medium by inoculating 10% seed culture. Cells were harvested by centrifugation at 15,000 g for 10 min and washed with 20 mM phosphate buffer (pH 7.0) twice.

Reagents

Validamycin A was provided by QianJiang Biochem. Co. Ltd., China. Validoxylamine A and valienamine were prepared according to the methods earlier described (Zheng et al., 2004, 2005, 2006). N-*p*-Nitrophenylvalidamine and N-*p*-nitrophenylvalienamine were prepared from validamine and valienamine according to Takeuchi's method (Takeuchi et al., 1985). *p*-Nitrophenyl-3-keto-validamine was prepared according to previous method (Zhang et al., 2007). All other chemicals used were of A.R. grade.

Preparation of crude enzyme

The washed cells were suspended in 10 ml of 50 mM Tris-HCl buffer (pH 7.0) and ruptured with sonication for 10 min with ice cooling. The supernatant resulting from centrifugation at 48,400 g for 1 h was used as the crude enzyme. Enzyme assay and protein assay were applied using this crude enzyme.

Enzyme assay

3-Keto-validoxylamine A C-N lyase activity was assayed with *p*-nitrophenyl-3-keto-validamine as the substrate due to its yellow color. The reaction mixture consisted of 0.1 ml of 10 mM substrate, 0.3 ml of 100 mM Tris-HCl buffer (pH 7.0) and 0.1 ml of enzyme solution, which was incubated at 40°C for 30 min. The enzyme products were analyzed by HPLC. HPLC was carried out with reversed C_{18} and acetonitrile-water (1:3, v/v) as the mobile phase. The products were determined by a UV absorption spectrophotometer at 398 nm. One unit of the 3-keto-validoxylamine A C-N lyase activity was defined as the amount of enzyme that caused the production of 1 µmol of p-nitroaniline per minute at pH 7.0, 40°C.

G3DH activity was assayed according the previous method (Zhang et al., 2006). One unit of the G3DH activity was defined as the amount of enzyme that caused the reduction of 1 µmol of DCPIP per min at pH 6.0, 25°C.

Protein assay

Protein quantitative analysis was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Crude enzyme reaction

10 ml of 0.1 M phosphate buffer (pH 6.0), 2 ml of 5 mM DCPIP solution and 2 ml of 5 mM N-p-nitrophenyl-validamine solution were added to a 50 ml vial and then 5 ml of crude enzyme was added to commence the reaction. The reaction was carried out at 30°C, and 100 rpm throughout this study. At appropriate intervals, 0.5 ml of the reaction mixture was sampled to determine the concentration of products by HPLC.

RESULTS AND DISCUSSION

Effect of carbon source

To investigate the effect of carbon source on the production of 3-keto-validoxylamine A C-N lyase and G3DH, *S. maltrophilia* was cultivated with 5 mg/ml various carbon sources for 24 h. The result is shown in Table. 1. The enzymatic activity of G3DH was 0.237 U/mg protein using validamycin A as carbon source, which was about 7 times than that of control (no carbon source was added). The enzymatic activity of G3DH was improved about 3 times by α-methyl-D-glucoside and 2 times by lactose at the same way. The enzymatic activity of 3-keto-valid-

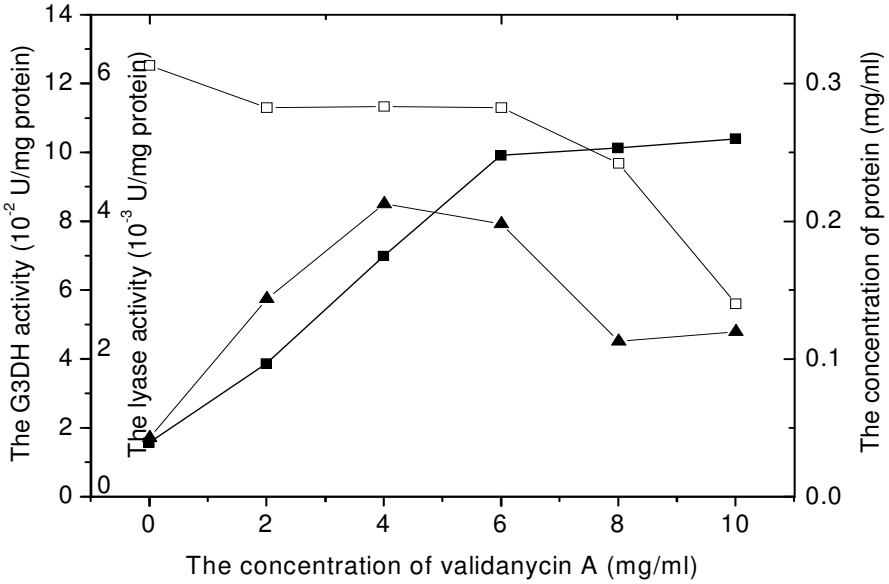

Figure 1. Effect of concentration of validamycin A on production of 3-keto-validoxylamine A C-N lyase (▲) and G3DH (■). □, The concentration of protein.

oxylamine A C-N lyase was 7.05 × 10^{-3} U/mg protein using validamycin A as carbon source, which was about fourfold of that achieved in the origin medium. Furthermore, the production of 3-keto-validoxylamine A C-N lyase was improved about 2 times by α-methyl-D-glucoside and lactose. Therefore, validamycin A is a perfect carbon source for both G3DH and C-N lyase production. Moreover, α-methyl-D-glucoside and lactose also increased these two enzymes production. So, validamycin A was used as carbon source for further study.

Since the bacterium was fermented to product valienamine using a minimal medium with validamycin A as a sole carbon source (Zheng et al., 2006), the cultivation of the bacteria was carried out using this minimal medium at first. The result indicated that both the 3-keto-validoxylamine A C-N lyase activity and G3DH activity were very low using this medium. On the other hand, validamycin A promoted the production of 3-keto-validoxylamine A C-N lyase and G3DH from *S. maltrophilia* at a high level using complex medium. In contrast, *F. saccharophilum* was cultivated at a media containing 0.5% meat extract, 1.5% polypeptone, 0.5% NaCl and 0.5% K_2HPO_4 (pH 7.0) for 3-keto-validoxylamine A C-N lyase and G3DH production (Takeuchi et al., 1985; Takeuchi et al., 1986). It is possible that 3-keto-validoxylamine A C-N lyase and G3DH of S. maltrophilia were probably different in enzyme production from that of *F. saccharophilum.*

Effect of validamycin A concentration

The effect of validamycin A concentration on the production of 3-keto-validoxylamine A C-N lyase and G3DH was investigated. *S. maltrophilia* was cultivated for 24 h in medium which contained 2, 4, 6, 8, 10 mg/ml or no validamycin A (control). The result is shown in Figure 1. The enzyme activity of G3DH was increased all along when the concentration of validamycin A was increased. At the first stage, from 0 to 6 mg/ml validamycin A, the enzyme activity was increased largely. Then, it was increased slightly. Whereas, the highest 3-keto-validoxylamine A C-N lyase production was achieved using the medium containing 4 mg/ml validamycin A and it was decreased slightly when the concentration of validamycin A was above 4 mg/ml. On the other hand, the concentration of protein declined when validamycin A was added. The reason was that validamycin A could inhibit bacteria's growth. Considering the enzyme activity and the growing organism, 4 mg/ml validamycin A was used for C-N lyase production and 6 mg/ml validamycin A was used for G3DH production.

Effect of volume in shaking culture

Since the fermentation volume was a key method to control dissolved oxygen in shaking culture, the effect of volume in shaking culture on the production of 3-keto-validoxylamine A C-N lyase and G3DH was examined at 40, 60, 80, 100, 120, 140 and 160 ml medium in a 500 ml conical flask for 24 h, respectively. As shown in Figure 2, the concentration of protein declined when the volume was up to 100 ml since the dissolved oxygen was not enough in this shaking culture condition. The specific activity of 3-keto-validoxylamine A C-N lyase and G3DH

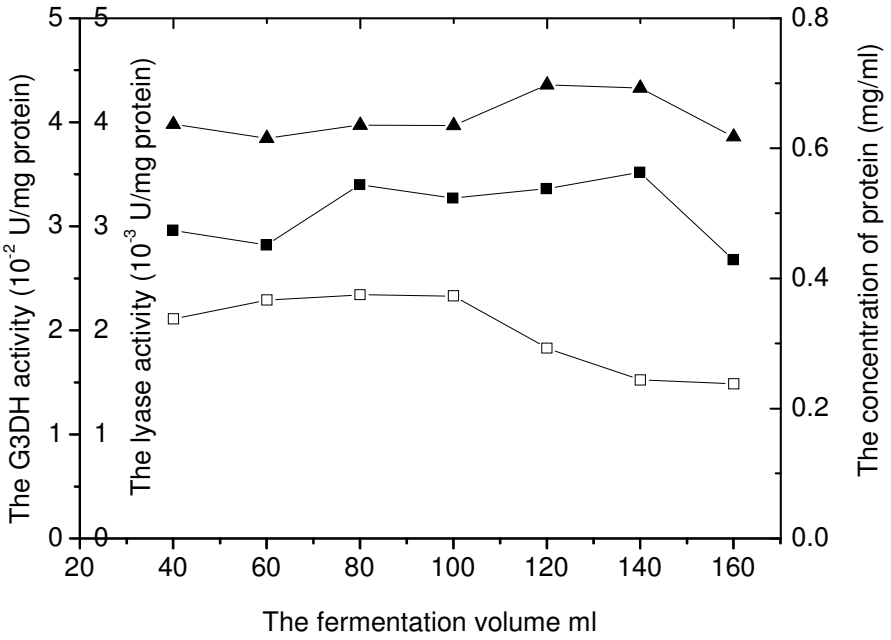

Figure 2. Effect of culture volume on 3-keto-validoxylamine A C-N lyase and G3DH production by *S. maltrophilia*. ▲,C-N lyase; ■,G3DH; □, The concentration of protein.

was affected by volume slightly. The specific activity of these two enzymes was decreased when the volume was up to 140 ml. In addition, the highest total activity of these two enzymes was achieved from 80 to 100 ml. In order to obtain more cells and harvest more enzymes, 100 ml culture medium into a 500 ml conical flask was used for further study.

Effect of seed volume inoculating

The effect of seed volume inoculating on the production of 3-keto-validoxylamine A C-N lyase and G3DH was examined at 2, 4, 6, 8, 10 and 12%, respectively. Likewise, inoculation from nutrient agar slants directly with one or two loop was also examined. As shown in Figure 3, the seed volume affected the enzymes production greatly. 3-Keto-validoxylamine A C-N lyase achieved the highest yield at seed volume of 10% and the highest specific activity of G3DH was achieved when 12% seed volume was inoculated. However, the total protein concentration was only slightly affected by seed volume from 4 to 12% and the highest protein concentration was obtained when 12% seed volume was inoculated. Moreover, the activity of these two enzymes was lower with inoculation from agar slants than that from fermentation solution. So 12% seed volume was used for further study.

Optimization

The optimal culture condition for 3-keto-validoxylamine A C-N lyase and G3DH producing was repeated at 100 ml culture medium into a 500 ml conical flask, with 5 mg/ml validamycin A as carbon source, at inoculation volume of 12%. The results were shown in Figure 4. The highest 3-keto-validoxylamine A C-N lyase activity was achieved at 22 h with a productivity of 6.26×10^{-3} U/mg protein. The specific G3DH activity was 0.244 U/mg protein at 27 h. However, the highest protein concentration was achieved at 47 h. So cells were harvested at 22-27 h for batch fermentation for further study. The enzyme activity of G3DH and 3-keto-validoxylamine A C-N lyase from *F. saccharophilum* was 34.3 U/mg protein (Takeuchi et al., 1986) and 0.71 U/mg protein (Takeuchi et al., 1985), respectively. According to Takeuchi's method, one unit of the G3DH activity was defined with the reduction of 1 nmol of DCPIP (Takeuchi et al., 1986), while it is defined as the reduction of 1 µmol of DCPIP in this paper. One unit of the lyase activity was defined per hour (Takeuchi et al., 1985), while it is defined per minute in this paper. Therefore, the G3DH activity of *S. maltrophilia* was higher than that from *F. saccharophilum* and the lyase activity of *S. maltrophilia* was as much as that of *F. saccharophilum*.

A 15 L fermenter (B, Braun Co. German) containing 8 liter of medium was used in this study. 5% 24 h old seed culture was inoculated to it for growth at 30°C. At intervals, the OD_{660} and the enzyme activity was detected. Dissolved oxygen was controlled at 4 vvm/L ventilation volume and the rotate speed was 200 rpm. As a result, the OD_{660} of culture comes to 1.69 at 36 h and the enzyme activity of G3DH and 3-keto-validoxylamine A C-N lyase was 0.231 U/mg protein and 8.16×10^{-3} U/mg protein at 27 h, respectively. At the other hand, the pH

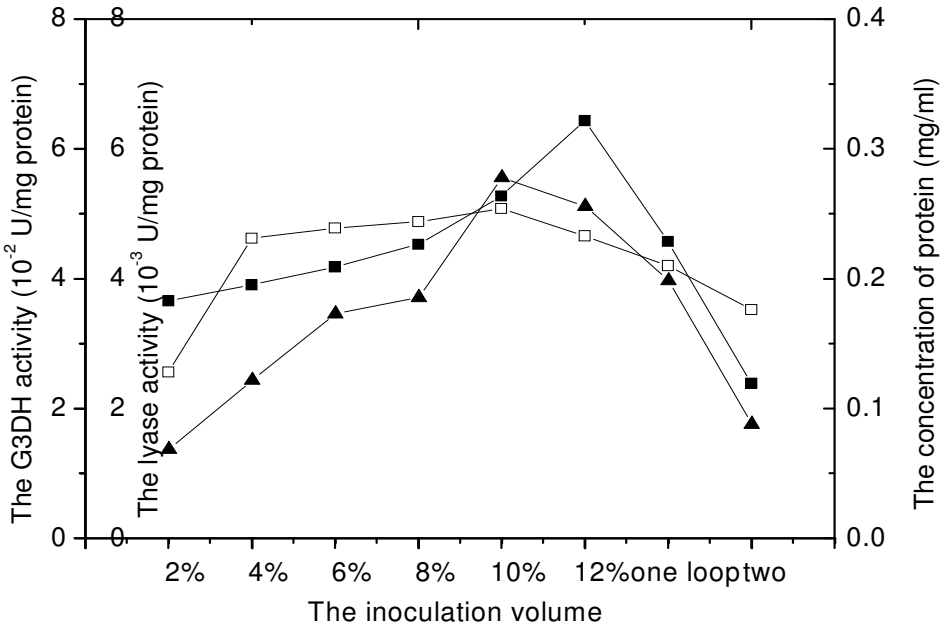

Figure 3. Effect of inoculation volume on 3-keto-validoxylamine A C-N lyase and G3DH production by *S. maltrophilia*. ▲,C-N lyase; ■,G3DH; □, The concentration of protein.

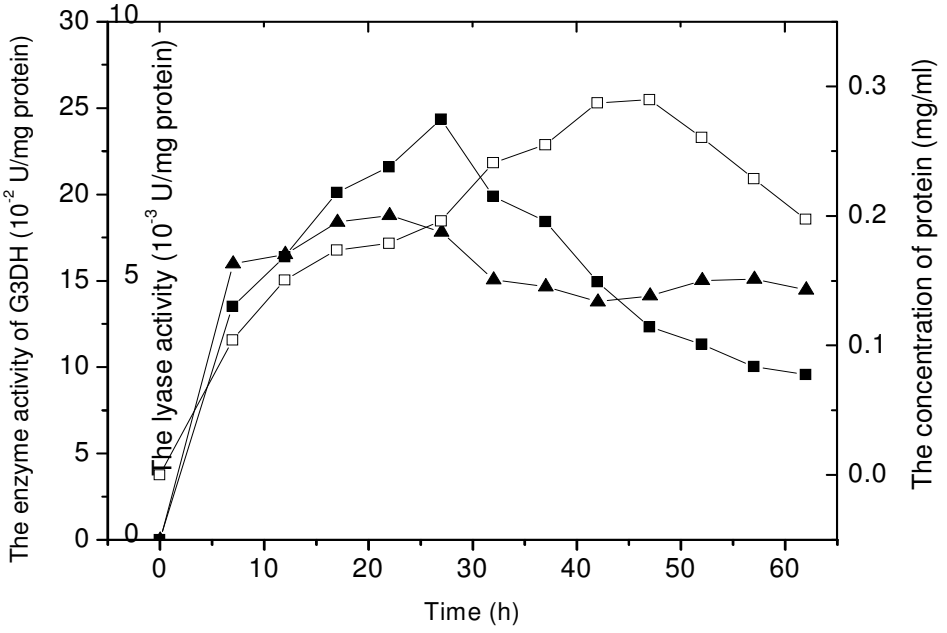

Figure 4. Effect of culture time on production of 3-keto-validoxylamine A C-N lyase and G3DH. ▲,C-N lyase; ■,G3DH; □, The concentration of protein.

value of fermentation solution was increased for almost the entire period of fermentation. The reason might be there were several basic products such as validamine and valienamine created, while validamycin A was exhausted in this process.

A lot of valienamine and validamine were detected from culture solution and validamycin A declined to zero at 45 h. After 27 h, both G3DH and C-N lyase activities declined. This result suggested that these enzymes producing were related to the concentration of validamycin A. However, the bacteria's growth could be inhibited by validamycin A. Therefore, high validamycin A concentra-

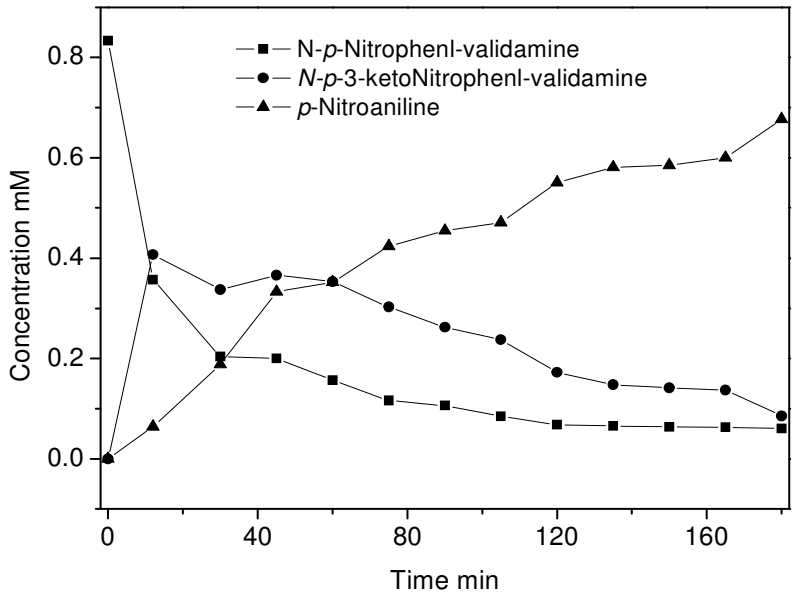

Figure 5. Enzyme reaction of N-p-Nitrophenylvalidamine by crud enzyme

tion at the initial phase will not be helpful. Further studies of fed-batch culture in fermenter, where the concentration of validamycin A will be kept at an adequate level, will improve the G3DH and 3-keto-validoxylamine A C-N lyase productivity.

Crude enzyme reaction

Since N-*p*-nitrophenyl-validamine can be cleaved at one position of C-N linkage and is easy to detect because of its yellow colour, it can be used as the substrate to study crude enzyme reaction. As shown in Figure 5, the concentration of N-*p*-nitrophenyl-validamine declined all through and the conversion of N-*p*-nitrophenylvalidamine increased to 92.8% after 3 h. At the same time, the concentration of *p*-nitroaniline increased and the yield of *p*-nitroaniline was 81.3% after 3 h. In addition, the concentration of N-*p*-nitrophenyl-3-ketovalidamine increased at the first 15 min, then it declined from 15 to 180 min. the yield of intermediate products, N-*p*-nitrophenyl-3-ketovalidamine, was only 10.2% after 3 h. Therefore, the enzyme production at this optimal culture condition was suitably for valienamine synthesis.

ACKNOWLEDGEMENTS

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REFERENCES

Asano N, Takeuchi M, Ninomiya K, Kameda Y, Matsui K (1984). Microbial degradation of validamyciNn A by *Flavobacterium Saccharophilum:* enzyme cleavage of C-N linkinge in validoxylamine Afr. J. Antibiot. 37(8): 859-867.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Chen XL, Fan YX, Zheng YG, Shen YC (2003). Properties and production of valienamine and its related analogues. Chem. Rev. 103: 1955-1977.

Kameda Y, Asano N, Yoshikawa M, Matsui K (1980). Valienamine as an α-glucosidase inhibition. J. Antibiot. 33: 1575-1576.

Kameda Y, Asano N, Yoshikawa M, Takeuchi M, Yamaguchi T, Matsui K (1984). Valiolamine, a new α-glucosidase inhibiting aminocyclitol produced by *Streptomyces hygroscopicus*. J. Antibiot. 37: 1301-1307.

Takeuchi M, Asano N, Kameda Y, Matsui K (1985). Purification and properties of 3-ketovalidoxylamine A C-N lyase from *Flavobacterium saccharophilum*. J. Biochem. 98: 1631-1638.

Takeuchi M, Asano N, Kameda Y (1986). Chemical modification by diethylpyrocarbonate of an essential histidine residues in 3-ketovalidoxylamine A C-N Lyase. J. Biochem. 99(6): 1571-1577.

Takeuchi M, Ninomiya K, Kawabata K, Asano N, Kameda Y, Matsui K (1986). Purification and properties of glucoside 3-dehydrogenase from *Flavobacterium saccharophilum*. J. Biochem. 100(4): 1049-1055.

Takeuchi M, Neyazaki K, Matsuim K (1990a). Chemical modification by 2,4,6-trinitrobenzenesulfonic acid (TNBS) of an essential amino group in 3-ketovalidoxylamine A C-N lyase. Chem. Pharm. Bull. 38(5): 1419-1420.

Takeuchi M, Asano N, Kameda Y (1990b). Fluorometric studies on the role of calcium in substrate binding to 3-ketovalidoxylamine A C-N lyase. Chem. Pharm. Bull. 36(9): 3540-3545.

Wang YS, Zheng YG, Shen YC (2007). Isolation and identification of a novel valienamine-producing bacterium. J. Appl. Microbiol. 102(3): 838-844.

Zhang JF, Zheng YG, Shen YC (2006). Purification and characterization of the glucoside 3-dehydrogenase produced by a newly isolated *Stenotrophomonas maltrophilia* CCTCC M 204024. Appl. Microbiol. Biotechnol. 71(5): 638-645.

Zhang JF, Zheng YG, Liu ZQ, Shen YC (2007). Preparation of 3-keto-

validoxylamine A C-N lyase substrate: N-p-nitrophenyl-3-ketovalidamine by *Stenotrophomonas maltrophilia* CCTCC M 204024. Appl. Microbiol. Biotechnol. 73(6): 1275-1281.
Zheng YG, Jin LQ, Shen YC (2004). Resin-catalyzed degradation of validamycin A for production of validoxylamine A. Catal. Commun. 5: 519-525.
Zheng YG, Shentu XP, Shen YC (2005). Inhibition of porcine small intestinal sucrase by valienamine. J. Enzyme Inhib. Med. Chem. 20: 49-53.
Zheng YG, Zhang XF, Shen YC (2005). Microbial transformation of validamycin A to valienamine by immobilized cells. Biocatal. Biotransform. 23: 71-77.
Zheng YG, Xue YP, Shen YC (2006). Production of valienamine by a newly isolated strain: *Stenotrophomonas maltrophilia.* Enzyme Microb. Tech. 39(5): 1060-1065.