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Significantly Enhanced Production of Acarbose in Fed-Batch Fermentation with the Addition of *S*-Adenosylmethionine

Sun, Li-Hui[1,2], Ming-Gang Li[1,2], Yuan-Shan Wang[1,2], and Yu-Guo Zheng[1,2]*

[1]*Institute of Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China*
[2]*Engineering Research Center of Bioconversion and Biopurification of the Ministry of Education, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China*

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Acarbose, a pseudo-oligosaccharide, is widely used clinically in therapies for non-insulin-dependent diabetes. In the present study, *S*-adenosylmethionine (SAM) was added to selected media in order to investigate its effect on acarbose fermentation by *Actinoplanes utahensis* ZJB-08196. Acarbose titer was seen to increase markedly when concentrations of SAM were added over a period of time. The effects of glucose and maltose on the production of acarbose were investigated in both batch and fed-batch fermentation. Optimal acarbose production was observed at relatively low glucose levels and high maltose levels. Based on these results, a further fed-batch experiment was designed so as to enhance the production of acarbose. Fed-batch fermentation was carried out at an initial glucose level of 10 g/l and an initial maltose level of 60 g/l. Then, 12 h post inoculation, 100 μmol/l SAM was added. In addition, 8 g/l of glucose was added every 24 h, and 20 g/l of maltose was added at 96 h. By way of this novel feeding strategy, the maximum titer of acarbose achieved was 6,113 mg/l at 192 h. To our knowledge, the production level of acarbose achieved in this study is the highest ever reported.

Keywords: Acarbose, *S*-adenosylmethionine, fed-batch, *Actinoplanes utahensis*

The α-glucosidase inhibitor acarbose, a widely available oral drug, has been widely used in the therapy of non-insulin-dependent diabetes mellitus owing to its good therapeutic and non-toxic effects. The drug was first launched in Germany in 1990 and has since been successfully marketed worldwide [5, 23]. Structurally, acarbose is a pseudo-oligosaccharide that consists of the two pseudo disaccharides, acarviosine and maltose. Acarviosine is composed of an unsaturated aminocyclitol connected with a 6-deoxy-D-glucose *via* an *N*-glycosidic bond [2, 14, 24]. The maltose unit in acarbose has been shown to be directly incorporated from maltose or maltotriose, rather than *via* the successive addition of glucose residues [12].

In recent years, the biosynthetic pathways, and related enzymes and genes for acarbose synthesis have been studied in depth and clearly described [16, 23]. However, major difficulties still exist in improving the yield of acarbose, leading to a high cost for its manufacture. Beunink *et al.* [1] found that not only low osmolalities (<200 mOsm/kg), but also high osmolalities (>600 mOsm/kg) could cause significantly lower acarbose production, and even completely inhibit its formation [1]. Choi and Shin [4] obtained a better acarbose yield of 3,490 mg/l at 500 mOsm/kg in the presence of 10 μmol/l of valienamine. Moreover, the by-product component C was seen to decrease by 90% to 43 mg/l when compared with the levels obtained in the absence of valienamine. Besides our own previous study [21], until the present time, the maximal acarbose titer in fermentation broth was obtained by Li *et al.* [13] at 4,327 mg/l in a 30 L fermentor by a fed-batch process, when controlling total sugar and reducing sugar at 75–80 and 45–50 g/l, respectively.

S-Adenosylmethionine (SAM), a ubiquitous substance in living organisms, is generally known to be a methyl donor in various biosynthetic processes. In previous studies, several researchers have documented that the external addition of SAM, or the overexpression of SAM synthetase, may increase the production of various secondary metabolites in actinomycetes, and possibly functions as a cofactor to provide methyl for methylation reaction, or as a signaling molecule to increase the transcription of pathway-specific regulatory genes [9, 11, 15, 19, 25]. These findings led us to assume that SAM may regulate the synthesis of acarbose

**Corresponding author*
Phone: +86-571-88320630; Fax: +86-571-88320630;
E-mail: zhengyg@zjut.edu.cn

in *Actinoplanes*, especially when considering the fact that streptomycin production was enhanced 1.35-fold by the supplementation of SAM at 1 mmol/l [17], and that both acarbose and streptomycin belong to the same family of aminoglycoside antibiotics [6].

We previously reported the isolation and identification of an acarbose-production mutant strain, *A. utahensis* ZJB-08196 [5, 22]. In the present work, we have attempted to determine the effect of the external addition of SAM on the production of acarbose, and excitingly, the results were in accordance with what we had anticipated. Moreover, the effects of glucose and maltose on the production of acarbose, both in batch and fed-batch processes, were investigated in detail. On the basis of these initial experiments, a reasonable strategy for fed-batch fermentation was designed so as to maximize acarbose production. To our knowledge, this is the first report that demonstrates the promise of the small molecule SAM in the regulation of acarbose production.

Materials and Methods

Microorganism, Media, and Cultivation

The mutant strain of *A. utahensis* ZJB-08196 was used for the production of acarbose in the present study [22]. This strain was kept in a 15% (v/v) glycerol stock solution and stored at −70°C. Before use, *A. utahensis* ZJB-08196 activation was carried out on agar plates at 27°C for about 8 days until visible orange colonies emerged. The medium for the agar plates was as follows (in g/l water): sucrose, 30; peptone, 2; L-Tyr, 1; K_2HPO_4, 1; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.1; and the initial pH value was adjusted to 7.0.

For inoculum, a colony of about a 1×1 cm^2 size, from a freshly prepared agar plate, was inoculated into a 500-ml shake flask containing 100 ml of seed medium and cultivated at 28°C and 200 rpm for 72 h. The seed medium consisted of (in g/l water) corn starch, 15; soybean flour, 40; glycerol, 20; $CaCO_3$, 2.0; K_2HPO_4, 0.5; and the initial pH value was adjusted to 6.7 with 6 mol/l NaOH prior to sterilization.

Batch and fed-batch fermentations were carried out by inoculating 10% (v/v) of the seed culture into 500 ml Erlenmeyer flasks containing 50 ml of fermentation medium at 28°C and 200 rpm for 168 h. The basal medium for acarbose fermentation was as follows (in g/l water): maltose, 43; soybean flour, 17; glucose, 40; sodium glutamate, 5; glycerol, 5; $FeCl_3 \cdot 6H_2O$, 0.83; $CaCO_3$, 2.5; K_2HPO_4, 1; $CaCl_2$, 1.88; and the initial pH value was adjusted to 7.0.

Exogenous SAM Treatment and Feeding Strategy for the Enhancement of Acarbose Production

Exogenous SAM treatment was applied to *A. utahensis* ZJB-08196 for the enhancement of acarbose production. Sterile-filtered aqueous solutions of SAM were added into the fermentation medium, and the effects of the added concentrations over time of SAM on acarbose production were investigated. Moreover, the effects of concentrations of glucose and maltose on acarbose production during batch and fed-batch processes were also studied. On the basis of the above experiments, an optimal feeding strategy, coupled with exogenous SAM addition, was performed for maximum acarbose production.

Analytical Methods

Samples (5 ml) were centrifuged at 9,000 ×*g* for 15 min in a pre-weighed tube. The cell precipitates were dried at 80°C to a constant weight for the determination of dry cell weight (DCW) as biomass. The supernatants were used for measuring the other fermentation parameters. Acarbose was analyzed by using a Shimadzu CT0-10ASVP HPLC system by methods previously described [22]. Glucose was estimated with a SBA-40E biosensor (Shandong, China). Maltose was measured according to known methodologies [10].

All experiments were performed three times in duplicate to ensure reliability and accuracy. The data reported herein are the mean ± standard deviation of the results.

Results and Discussion

Impact of Exogenous Addition of SAM on Acarbose Production in Batch Culture

The exogenous addition of SAM to the culture medium was performed with the final SAM concentration range

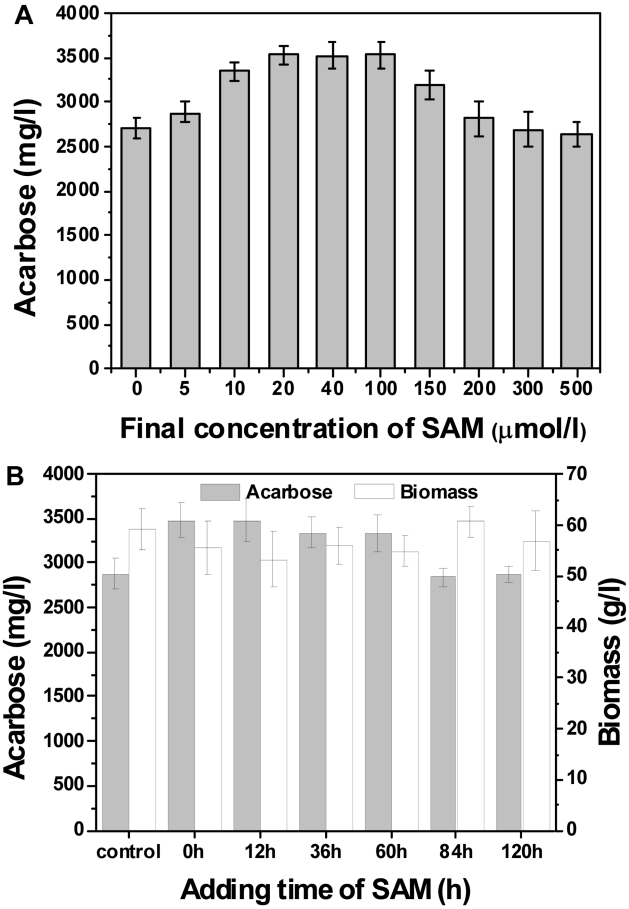

Fig. 1. Effects of (**A**) adding dosage and (**B**) adding time of SAM on acarbose production by *A. utahensis* ZJB-08196.
Batch fermentation was performed in basal media with (**A**) the exogenous addition of SAM in the range from 5 to 500 μmol/l at 12 h post inoculation, and (**B**) the exogenous addition of SAM of 100 μmol/l during cultivation (0–120 h).

being between 5 and 500 μmol/l. As shown in Fig. 1A, SAM exhibited a stimulating effect on acarbose production in the concentration range of 10 to 150 μmol/l, whereas in high concentrations of more than 500 μmol/l, it exerted a negative impact (data not shown). Adding concentrations of SAM of 20 to 100 μmol/l was more suitable for acarbose production, as this was seen to provide about 30% higher concentrations of acarbose than the control. The effects of SAM over time on acarbose production are illustrated in Fig. 1B. The results revealed that the addition of SAM was only favorable for the production of acarbose when SAM was added before the stationary phase.

In previous studies, the exogenous addition of SAM, and/or the overexpression of SAM synthetase, has been proven to activate secondary metabolism directly or indirectly in *Streptomycetes*. SAM can also be used as a methyl donor for methylation reactions [25], as a signaling molecule to increase the transcription of pathway-specific regulatory genes [11, 15, 19] or to increase the autophosphorylation of a regulatory kinase [26]. Huh *et al.* [9] have found that the production of four antibiotics could be increased notably by the addition of SAM in the culture media. They further demonstrated that the transcription of *gra*-ORF9, which regulates the biosynthesis of granaticin, could be activated significantly by exogenous SAM treatment at 48 h. Moreover, by studying the effects of adding methyltransferase inhibitor, sinefungin and *S*-adensylhomocysteine (SAH) on production, it has been pointed out that SAM may act as both a methyl donor and an intracellular factor in the biosynthesis of oleandomycin and avermectin [9]. Shin *et al.* [20] have reported that SAM can induce several ATP-binding cassette (ABC) transporters to modulate secondary metabolism in *Streptomyces coelicolor*. While in the process of acarbose production, one methylation reaction was observed in the conversion step of dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose [23]. In addition, the ABC transporters are also important for maltose and maltotriose transport in the biosynthesis of acarbose [3]. Owing to the fact that SAM may be involved in many aspects of cellular physiology to regulate secondary metabolism, it is difficult to determine the real mechanism involved in the production of acarbose. It is clear that the stimulating mechanism of the exogenous addition of SAM on the increase in acarbose production should be further explored.

Effects of Glucose Concentrations on the Process of Acarbose Fermentation

Complex carbon sources consisting of glucose and maltose are the preferable carbon sources for cell growth and acarbose biosynthesis [4, 13, 22]. Glucose has always been regarded as the most easily utilizable carbon source. However, high concentrations of glucose are capable of exerting repression on the biosynthesis of secondary metabolism [7, 8, 18]. In order to select an optimal glucose concentration for acarbose production, batch fermentation in flasks was performed at initial glucose concentrations ranging from 10 to 70 g/l (Fig. 2). The maximal acarbose titer of 3,213 mg/l occurred at 50 g/l glucose at 168 h. However, high acarbose production levels were observed when relatively low glucose levels in the early stages of fermentation were applied, and the lower the initial glucose level used, the higher the specific acarbose production rate was noted in the early stages. The maximal acarbose titer of 780 mg/l was obtained in the first 48 h when 10 g/l of glucose was used in the medium. After that, the glucose supply was completely exhausted (Fig. 2B), which resulted in a decrease in the acarbose formation rate, and even in a decrease of acarbose titer after 120 h. In addition, we found that maltose was consumed slowly in the first 72 h,

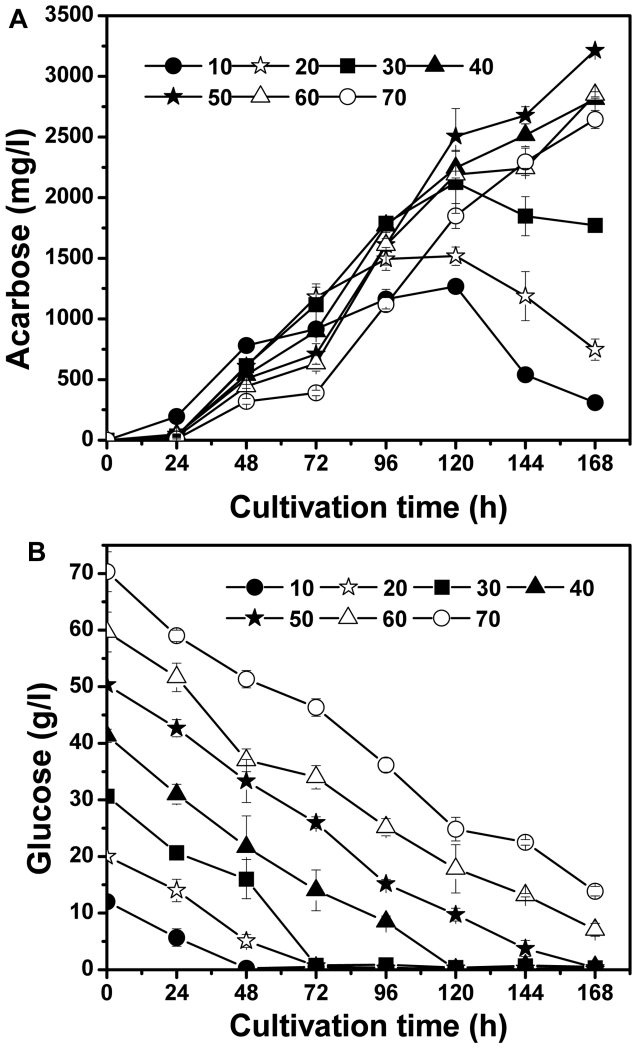

Fig. 2. Effects of initial glucose concentrations on acarbose production by *A. utahensis* ZJB-08196.
(**A**) Acarbose titer; (**B**) glucose concentration. Batch fermentation was performed in an initial glucose concentration range of 10 to 70 g/l, and other nutritional components were in accord with the basal fermentation media.

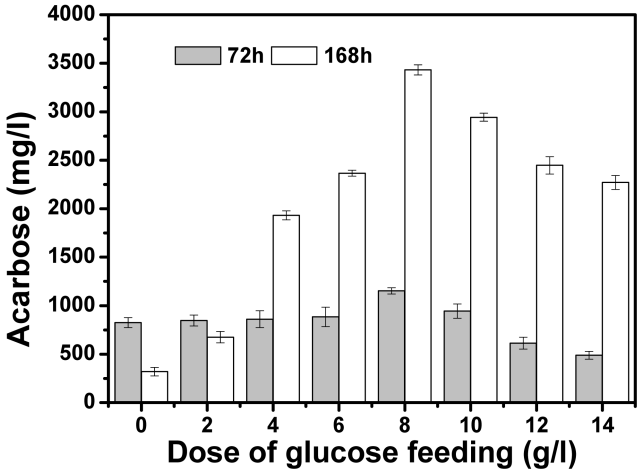

Fig. 3. Dose of glucose, fed once every 24 h, on acarbose production at 72 h and 168 h.
Fed-batch fermentation was carried out with an initial 10 g/l of glucose, and then a different dose of glucose was added to the media once every 24 h. Other nutritional components were in accord with the basal fermentation media.

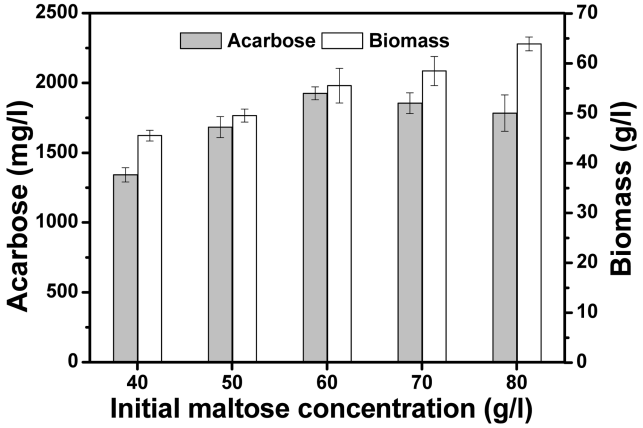

Fig. 4. Effects of initial maltose concentrations on acarbose production and *A. utahensis* ZJB-08196 growth at 72 h.
To investigate the effect of initial maltose concentration on acarbose production, fed-batch fermentation was carried out with 10 g/l of initial glucose, and additionally fed glucose of 8 g/l once every 24 h, with an initial maltose range from 40 to 80 g/l. Other nutritional components were in accord with the basal fermentation media.

after which time it was consumed dramatically (data not shown), accompanied by the rapid accumulation of acarbose. These phenomena could be explained by two hypotheses. One reason might be that maltose was utilized as the carbon source for cell growth because of the exhaustion of glucose. On the other hand, maltose could have been used as the direct precursor for the biosynthesis of acarbose [4], which resulted in its reduction in the mid-late stage of fermentation.

Glucose exhaustion during the early growth stages could have caused low maltose levels during later stages, which may have led to the reduction of acarbose production in the broth. These findings are in general agreement with a previous report [4]. From the above results, it could be concluded that 10 g/l of initial glucose was the most advantageous for acarbose production, but that its effect was reduced to close to zero by 48 h. Therefore, it was surmised that the addition of glucose should be controlled and fed periodically in limited amounts during the fermentation process. Fed-batch fermentations were carried out with an initial glucose concentration of 10 g/l, and then glucose of 0 to 12 g/l were supplemented once every 24 h. As shown in Fig. 3, the culture with a glucose supplementation of 8 g/l was the most effective for the increase of acarbose titers, both in the middle and end stages of fermentation. Acarbose production decreased when glucose was fed above 10 g/l or below 6 g/l every 24 h.

Effects of Maltose Concentrations on the Process of Acarbose Fermentation

Concentration levels of maltose play very important roles in increasing acarbose production. A previous study has shown that the maltosyl unit of acarbose is mainly derived directly from maltose based on *Actinoplanes* sp. cultures using [^{14}C-]maltose [12]. In addition, a high maltose level is favorable for acarbose production, as it contributes towards the establishment of an appropriate osmolality environment [1, 4, 10]. Thus, further experiments had to be performed in order to determine the optimal maltose concentration for acarbose fermentation. Based upon the experimental results above (Fig. 2 and 3), fed-batch cultures, with the initial maltose concentration varied from 40 to 80 g/l, were carried out to investigate the effect of maltose on acarbose production. As shown in Fig. 4, the biomass increased with increasing initial maltose concentrations.

Table 1. Strategy for maltose feeding on the acarbose titer and *A. utahensis* ZJB-08196 growth at 168 h.

No.	Adding strategy of maltose			Acarbose titer (mg/l)	Biomass (g DCW/l)
	96 h	120 h	144 h		
1	10 g/l	--	--	4,202 ± 232	52.8 ± 3.1
2	15 g/l	--	--	4,543 ± 70	54.3 ± 2.0
3	20 g/l	--	--	5,022 ± 203	67.4 ± 0.4
4	25 g/l	--	--	4,740 ± 153	66.9 ± 0.2
5	30 g/l	--	--	4,503 ± 123	64.0 ± 2.7
6	4 g/l	4 g/l	4 g/l	3,466 ± 251	48.3 ± 1.9
7	8 g/l	8 g/l	8 g/l	3,776 ± 126	51.4 ± 2.9
8	12 g/l	12 g/l	12 g/l	4,354 ± 211	49.2 ± 3.3
9	14 g/l	14 g/l	14 g/l	4,631 ± 317	52.5 ± 3.9
10	20 g/l	20 g/l	20 g/l	4,915 ± 156	58.4 ± 2.1

To investigate the effects of maltose feeding modes on acarbose production, fed-batch fermentation was carried out with an initial 10 g/l of glucose and 60 g/l of maltose. Further glucose of 8 g/l was fed once every 24 h. Other nutritional components were in accord with the basal fermentation media.

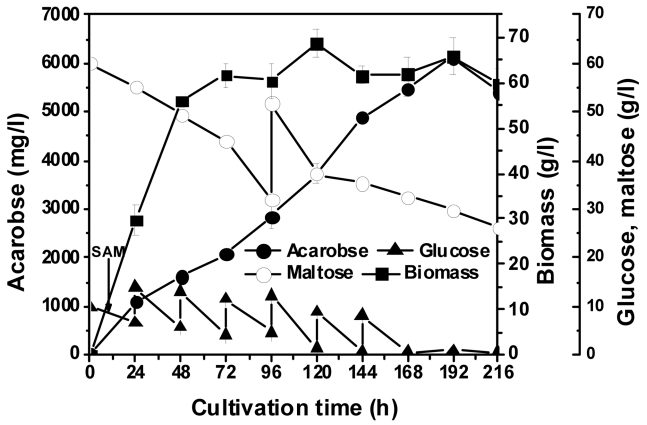

Fig. 5. Time course of acarbose titer, biomass, glucose, and maltose during fed-batch cultivation of *A. utahensis* ZJB-08196 with an optimized feeding strategy.
Symbols: acarbose titer (●); biomass (■); glucose (▲); maltose (○). Fed-batch fermentation was carried out with 10 g/l of initial glucose and 60 g/l of initial maltose, with the external addition of 100 μmol/l of SAM at 12 h post inoculation, and the additional feeding of 8 g/l glucose once every 24 h, with the feeding of 20 g/l maltose at 96 h.

However, the maximum acarbose titer of 1,926 mg/l was obtained in 72 h when an initial maltose of 60 g/l was used. These results might indicate that acarbose fermentation by *A. utahensis* ZJB-08196 is in the category of partially growth-associated, which has also been theorized by Wang *et al.* [22].

Considering the rapid utilization of maltose after 72 h, different patterns of maltose feeding were carried out. As shown in Table 1, the maximum concentration of acarbose achieved was 5,022 mg/l in a fed-batch culture by maltose supplementation of 20 g/l at 96 h. As mentioned above, low concentrations of maltose at later stages could result in relatively low acarbose production owing to the decrease of osmolality. Thus, it is more important for acarbose production to maintain maltose at an appropriate level during cultivation.

A Strategy for Maximizing Production of Acarbose in Fed-Batch Fermentation with the Exogenous Addition of SAM

Based on the above results, it was seen that initial glucose at a relatively low level (10 g/l) and initial maltose at a relatively high level (60 g/l) were the most favorable conditions for acarbose production. In order to enhance acarbose production, effective supplements of glucose and maltose are very necessary during the culture process. Meanwhile, the above results demonstrated that it was preferable, for the production of acarbose, to add an appropriate content of SAM into the medium before the stationary phase. Therefore, a new fed-batch experiment was designed for the enhanced production of acarbose (Fig. 5). An improved strategy of feeding sugars intermittently, under optimized conditions, was adopted, with the external addition of 100 μmol/l SAM at 12 h. In this way, it was hoped that the inhibitory effects of the sugars on biosynthesis and cell growth, could be avoided. Finally, it was shown that, *via* a suitable fed-batch strategy, acarbose titer was increased about 1-fold when compared with that a batch culture without the external addition of SAM.

In conclusion, this study examined the effects of glucose, maltose, and the external addition of SAM on the production of acarbose and has proposed a reasonable strategy for fed-batch fermentation to achieve a high acarbose titer. It should be pointed out that the stimulating effect of the external addition of SAM on acarbose production is the first such report to date. The mechanism of the exogenous addition of SAM to increase acarbose production should be further explored. By adopting this novel feeding strategy, the maximum titer of acarbose observed was 6,113 mg/l at 192 h. To our knowledge, the production level of acarbose noted through this study is the highest ever reported.

Acknowledgments

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