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Research Article

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Optimization of carbon source for the biosynthesis of *α*-amylase inhibitors by *S.coelicoflavus* ZG0656

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ABSTRACT

In this study, the fed-batch fermentation technique was applied to improve the yield of α-amylase inhibitors produced by S.coelicoflavus ZG0656. Various fermentation substrates and conditions were investigated to identify the optimal concentration of carbon source in the production of α-amylase inhibitors. The ratio of maltose and glucose were found to be the optimal initial carbon source, and the optimal ratio containing 3:1 (mass ratio) of maltose and glucose and its optimal concentration was determined to be 100 g/L based on the results of fermentations conducted in a 5-L jar fermenter using a series of fed-batch cultures of S.coelicoflavus ZG0656. The effects of mixed carbon sources concentration and medium osmolality on the production of α-amylase inhibitors were also investigated in this work. Our results showed that the production of α-amylase inhibitors by S.coelicoflavus ZG0656 was enhanced when the feeding medium containing 2:1(mass ratio) of maltose and soluble starch and 70-80 g/L of total sugar. Under the optimal conditions, a final α-amylase inhibitors concentration of 4726 mg/L was achieved after 168 h.

Keywords: *S.coelicoflavus* ZG0656, *α*-amylase inhibitors, Fermentation, Carbon source, Optimization.

INTRODUCTION

Saccharide hydrolase inhibitors, such as amylase and glucosidase inhibitors, are well-known as treatments and prophylactics for diabetes, hyperlipoproteinemia, hyperlipidemia, obesity, or other secondary symptoms caused by these diseases [1]. In the course of previous screening for novel *α*-amylase inhibitors, Bai, G et al [2] discovered a series of compounds secreted by *S.coelicoflavus* ZG0656, termed acarviostatins. This family of secondary metabolites consists of acarviosine-containing aminooligosaccharides, which show remarkable inhibitory activity against porcine pancreatic *α*-amylase.

So far, commercial production of *α*-amylase inhibitors is exclusively via microbial fermentation with strains from the genera of *Actinoplanes*. Many experiments including producer strain mutagenesis and screening, media formula, fermentation conditions, and *α*-amylase inhibitors isolation and purification have been conducted in order to increase *α*-amylase inhibitors yield because of its high commercial [3-6]. However, major difficulties still exist in improving the yield of acarbose, leading to a high cost for its manufacture. Recently, Li et al. [7-8] reported the medium optimization and scale-up strategy for acarbose fermentation by *Actinoplanes* sp. A56 and developed an optimized industrial fermentation processes for acarbose production, as a result about 5000 mg/L of acarbose was obtained. Our group has extensively studied the production of acarbose [9-13]. A high acarbose-producing mutant stain *A. utahensis* ZJB-08196 was isolated by mutagenesis and screening method [9]. Fed-batch fermentation with *A. utahensis* ZJB-08196 at elevated osmolality via intermittently feeding of necessary components regarding acarbose formation afforded a peak acarbose titer of 4878 mg/L [10].

In the present work, the effects of mixed carbon sources concentration and medium osmolality on the production of *α*-amylase inhibitors by *S.coelicoflavus* ZG0656 were investigated in detail in 5-L jar fermenter.

EXPERIMENTAL SECTION

Microorganism

S.coelicoflavus strain ZG0656, which was collected from soil at the Nankai University campus, Tianjin, China, in 2005, was identified by the Department of Microbiology, Nankai University. The strain (CGMCC 2097) was deposited in China General Microbiological Culture Collection Center, Institute of Microbiology, Academia Sinica.

Medium

Agar slants containing (g/L): glucose, 20; peptone, 5; KCl, 0.5; K_2HPO_4, 1.0; $MgSO_4$, 0.5; agar, 20. The pH was adjusted to 7.0 with 1 M NaOH prior to sterilization.

Inoculum medium was composed of (g/L): starch, 10; glucose, 20; corn steep liquor, 20; soybean flour, 10; K_2HPO_4, 1.0; $MgSO_4$, 1.0; $CaCO_3$, 20. The pH was adjusted to 7.0-7.2 with 1 M NaOH Prior to sterilization.

The fermentation medium contained the following ingredients (g/L): carbon sources, corn steep liquor, 10; soy bean flour, 20; monosodium glutamate, 1.0; $FeCl_3$, 0.5; K_2HPO_4, 1.0; $CaCO_3$ 2.0. The pH was adjusted to 7.2-7.4 with 1 M NaOH before autoclaving.

Culture conditions

A single colony of *S.coelicoflavus* ZG0656 was inoculated to a 500 mL baffled flask containing 30mL growth media and was cultivated at 28 ℃ and 150 rpm for 48 h. This seed culture was then transferred into a fermenter.

The fed-batch fermentations were performed in a 5-L jar fermenter (BioFlo® 115 Fermenter System, New Brunswick Scientific, Edison, NJ, USA) containing 3L of fermentation medium. The inoculation size was 15% (v/v). Data logging and operational parameters were controlled by the BioCommand Plus BioProcessing Software (New Brunswick Scientific).

Then the seed culture (450 mL) was transferred into a 5-L jar fermenter with 3 L of fermentation medium. The pH of the fermentation medium was adjusted to 7.0-7.2 before inoculation. During the fermentation process of 168 h, the temperature was controlled at 28 ℃, and the dissolved oxygen (DO) was kept at about 30% by adjusting agitation speed and airflow rate. When the reducing sugar in the broth dropped to 40-50 g/L, feeding medium was added to keep the total sugar and reducing sugar maintain at about 70-80 and 40-50 g/L, respectively.

Analytical Methods

Dry cell weight was gravimetrically determined using the pellet fraction from the 10 samples. After centrifugation at 13000 r/min for 20 min and washing twice with distilled water, the biomass was poured into preweighed aluminum cups and placed in a ventilating oven at 80℃ overnight until constant weights were obtained [14]. Maltose was measured according to know methodologies [15]. Soluble starch was measured according to know methodologies [16]. Glucose in broth was measured with SBA-40E biosensor (Biology Institute of Shandong Academy) after dilution (10-100X) with deionized water. *α*-amylase inhibitors measured according to know methodologies [17].

RESULTS AND DISCUSSION

Effects of initial carbon sources on *α*-amylase inhibitors production

In order to find the optimal carbon source of *α*-amylase inhibitors production by *S.coelicoflavus* ZG0656, different types of sucrose, glucose, maltose, soluble starch and dextrin were added to the fermentation media at 80 g/L using 5-L jar fermenter. The yields of *α*-amylase inhibitors produced from various carbon sources were determined after 100 h of cultivation in 5-L jar fermenter. The dry cell weight and the yield of *α*-amylase inhibitors in each case are shown in Fig.1.

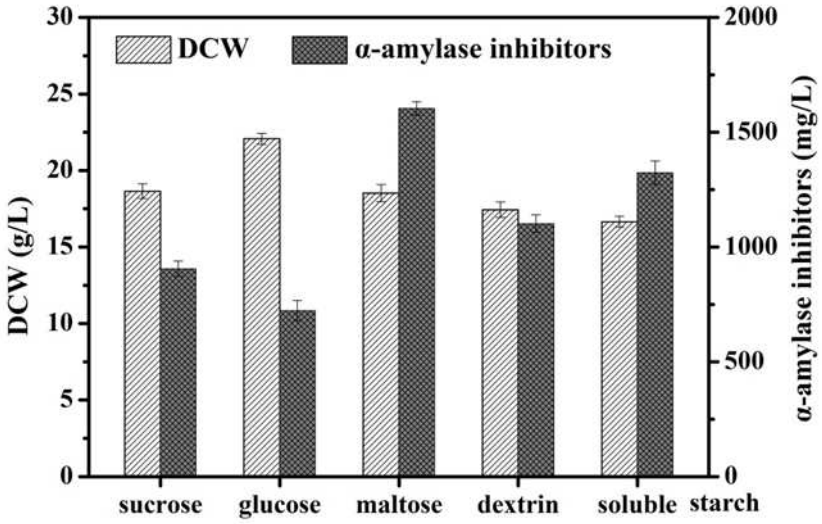

Fig.1 Effects of initial carbon sources on *α*-amylase inhibitors production

It is generally accepted that bacteria can grow to various extents in a wide range of carbon sources [18]. As shown in Fig.1 that glucose could improve cell growth but significantly restrain *α*-amylase inhibitors biosynthesis, and maltose was optimum carbon source for *α*-amylase inhibitors production. Possibly due to the fact that *S.coelicoflavus* ZG0656 was able to uptake sugar and turned on glycolysis immediately after sugar was transported into the cell, but the single carbon source of glucose bring about catabolite repression, while maltose has been reported to directly incorporate into acarbose molecules, acting as the precursor [19], and significantly affected of *α*-amylase inhibitors biosynthesis. After 100 h of cultivation, *α*-amylase inhibitors titer reached the maximum value of 1604 ± 19.3 mg/L.

Effects of mixed carbon sources on *α*-amylase inhibitors production

According to the results presented in Fig. 1, maltose stimulated *S.coelicoflavus* ZG0656 produce *α*-amylase inhibitors, in order to determine the optimal initial mixed carbon source for *α*-amylase inhibitors production, on the basis of maltose as the initial medium carbon source with different carbon source were performed to investigate the effect of the mixed carbon source for *α*-amylase inhibitors production. The ratio of maltose and other one carbon source was 1:1 and the total sugar of broth was 80 g/L. The results were shown in Fig.2.

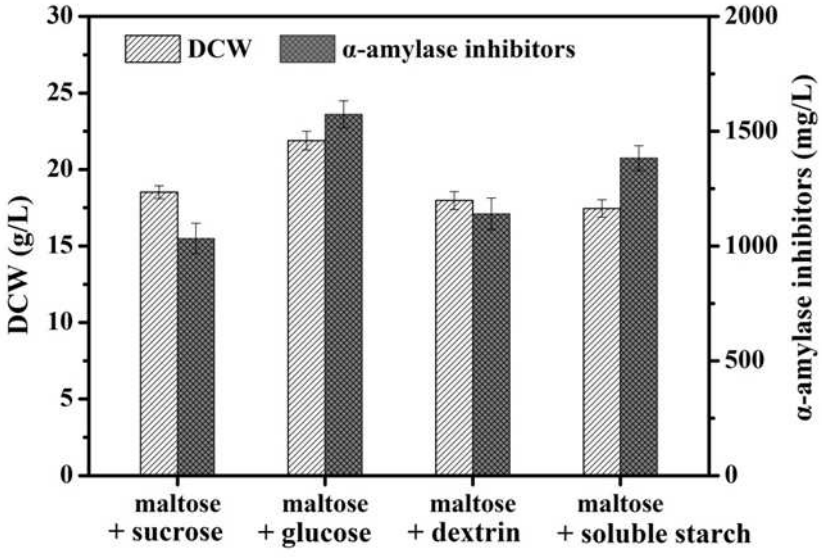

Fig.2 Effect of mixed carbon sources on *α*-amylase inhibitors production

The dry cell weight and the yield of *α*-amylase inhibitors in different mixed carbon source are plotted in Fig.2. With composite carbon source composed of glucose and maltose, *S.coelicoflavus* ZG0656 exhibited a relatively high cell growth and the weight of *α*-amylase inhibitors produced, the *α*-amylase inhibitors yield reached the maximum value of 1574 ± 17.93 mg/L, whereas with other mixed carbon source, the yield of *α*-amylase inhibitors and the dry cell weight is relatively lower comparing to the former. Maltose in the fermentation medium was used as an energy source and as a precursor for *α*-amylase inhibitors. It was found that maltose was consumed slowly in the early stage of fermentation. After glucose was exhausted, maltose was consumed dramatically, accompanied by the rapid accumulation of *α*-amylase inhibitors. It was utilized not only as the carbon source for cell growth because of the exhaustion of glucose, but also as the direct precursor for the biosynthesis of *α*-amylase inhibitors.

Effects of the ration of maltose and glucose in initial medium on *α*-amylase inhibitors production

According to the biosynthetic pathway of acarbose, maltose and glucose directly incorporated into acarbose [20]. Therefore, the ratio of maltose and glucose in fermentation broths probably had an important role in *α*-amylase inhibitors biosynthesis. To explore the appropriate ratio of maltose and glucose production in *S.coelicoflavus* ZG0656, the initial medium was consisted of eight various mass ratios of maltose and glucose (0:1, 1:0, 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6). The experimental results and their analysis of variance are listed in Table 1.

Table 1 Effect of the ration of maltose and glucose in initial medium on *α*-amylase inhibitors production

M:G[a]	DCW (g/L)	*α*-amylase inhibitors (mg/L)
0:01	22.08 ± 0.06	723 ± 24.01
1:00	18.52 ± 0.16	1604 ± 19.30
1:01	21.89 ± 0.31	1574 ± 17.93
2:01	21.03 ± 0.06	1856 ± 19.30
3:01	19.54 ± 0.16	2163 ± 21.58
4:01	19.46 ± 0.50	1783 ± 18.24
5:01	19.34 ± 0.21	1692 ± 35.12
6:01	19.07 ± 0.33	1601 ± 19.37

[a] *Mass ratio of maltose and glucose in the feeding medium*

As shown in Table 1, the ratio of maltose and glucose had a significant effect on the *α*-amylase inhibitors biosynthesis. In the case of solely using maltose or glucose as the carbon source in initial medium, only 1604 ± 19.3 and 723 ± 24.01 mg/L of *α*-amylase inhibitors were obtained. When a 3:1 ratio of maltose and glucose was add to the fermentation broth, the maximum *α*-amylase inhibitors (2163 ± 21.58 mg/L) was achieved. Therefore, based on the results and their multiple comparisons, it could be concluded that the initial medium with a 3:1 ratio of maltose and glucose was favorable for *α*-amylase inhibitors biosynthesis in *S.coelicoflavus* ZG0656.

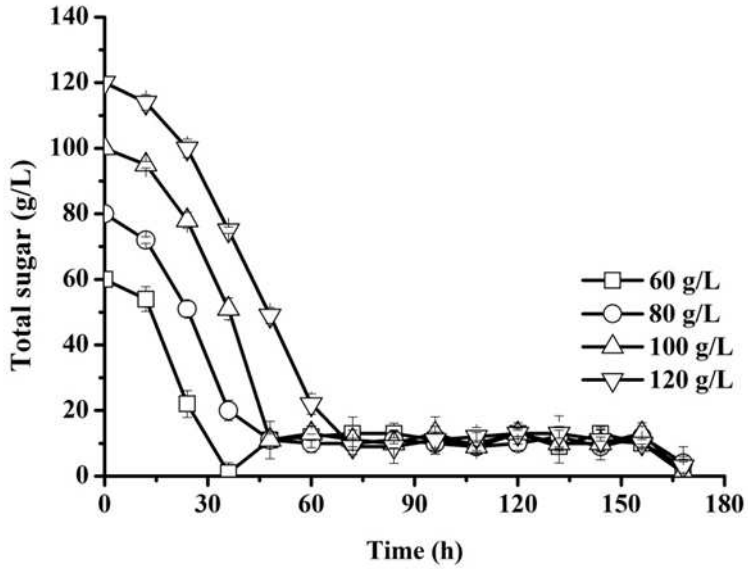

Fig.3a Total sugar consumption by *S.coelicoflavus* ZG0656 at different initial concentrations

Effects of total sugar concentration on *α*-amylase inhibitors production

In order to determine the optimal initial total sugar concentration for *α*-amylase inhibitors production, four fed-batch cultures were evaluated with media containing different concentrations of total sugar. After total sugar depletion, an 800 g/L glucose solution was fed into the jar fermenter until the total sugar in the media reached approximately 10

g/L. Total sugar consumption by *S.coelicoflavus* ZG0656 at different initial concentrations is shown in Fig. 3a. With high initial total sugar concentration, total carbon sources uptaken was slow in the lag phase of cultivation, but became fast after 18 h. When initial total sugar concentrations were 60 and 80 g/L, The total sugar depleted at 36 and 44 h, respectively. When initial total sugar concentration was increased from 100 to 120 g/L, the depletion time was increased from 54 and 68 h accordingly.

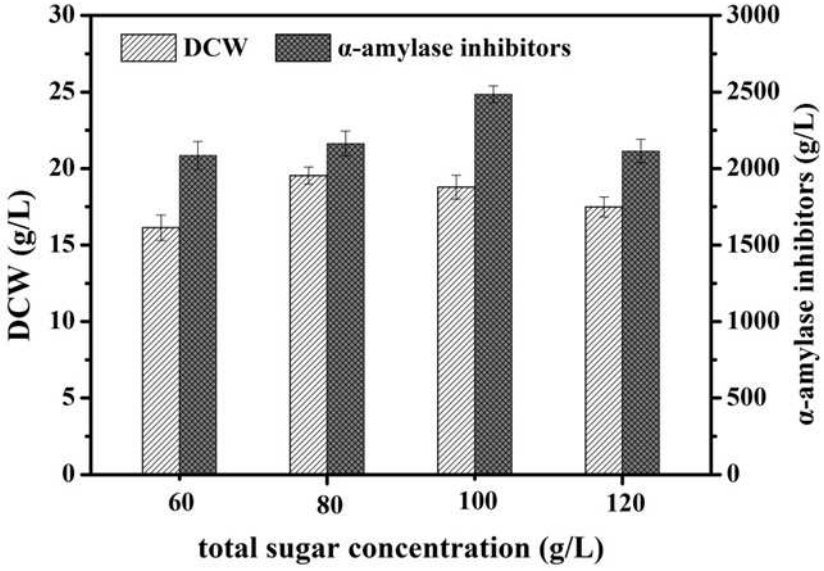

Fig.3b Effects of total sugar concentration on *α*-amylase inhibitors production

The dry cell weight and the yield of *α*-amylase inhibitors in each case are shown in Fig.3b, a downtrend of biomass could be observed along with the increase of total sugar concentration, which revealed that a too high concentration of total sugar had a significantly negative effect on cell growth of *S.coelicoflavus* ZG0656 Fig.3b showed the kinetics of *α*-amylase inhibitors production under the three fermentation runs, and the peak *α*-amylase inhibitors production were 2085 ± 21.31, 2163 ± 21.58, 2485 ± 24.56 and 2113 ± 17.24 g/L, respectively. Due to 120 g/L of total sugar concentration inhibiting cell growth, the lowest *α*-amylase inhibitors production was consequently obtained. Noticeably, although the biomass under 80 g/L of total sugar was much higher than that obtained in the case of 100 g/L of total sugar, the *α*-amylase inhibitors production markedly presented lower.

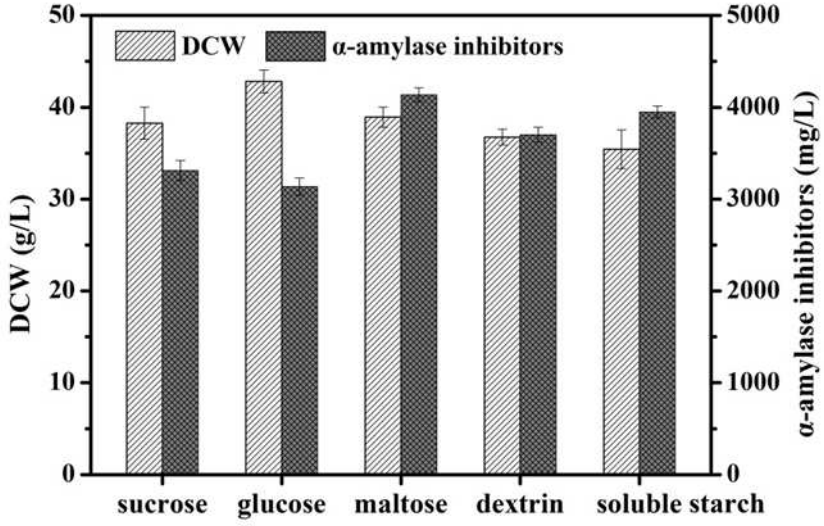

Fig.4 Effects of carbon sources in feeding medium on *α*-amylase inhibitors production

Effects of carbon sources in feeding medium on *α*-amylase inhibitors production

In order to find the optimal carbon source of *α*-amylase inhibitors production by *S.coelicoflavus* ZG0656, different

types of sucrose, glucose, maltose, soluble starch and dextrin were added to the feeding media at 60 g/L using 5-L jar fermenter. The yields of *α*-amylase inhibitors produced from various carbon sources were determined after 168 h of cultivation in 5-L jar fermenter. The dry cell weight and the yield of *α*-amylase inhibitors in each case are shown in Fig. 4.

As shown in Fig.4 that maltose and soluble starch was optimum carbon source for *α*-amylase inhibitors biosynthesis, and glucose could improve cell growth but significantly restrain *α*-amylase inhibitors production. Five *α*-glucosidic hydrolase and/or glycosyltransferases are biosynthetic in *S.coelicoflavus* ZG0656 [21], three extracellular *α*-amylases are responsible for the degradation of amylases or soluble starch in the environment. Furthermore, these three enzymes may also have transglycosylation activities, which encourage us to speculate the extracellular assemblies of acarviostatin homologues. To synthesize the major products of *S.coelicoflavus* ZG0656 that acarviostatin homologues might act as 'pseudotrisaccharide-transferases (TSTases)', which have maximum affinities to trisaccharide moieties.

Effects of mixed carbon sources in feeding medium on *α*-amylase inhibitors production

According to the results presented in Fig.4, maltose stimulated *S.coelicoflavus* ZG0656 produce *α*-amylase inhibitors, in order to determine the optimal feeding mixed carbon source for *α*-amylase inhibitors production, on the basis of maltose as the feeding medium carbon source with different carbon source were performed to investigate the effect of the mixed carbon source for *α*-amylase inhibitors production. The ratio of maltose and other one carbon source was 1:1 and the total sugar of broth was 60 g/L. The results were shown in Fig.5.

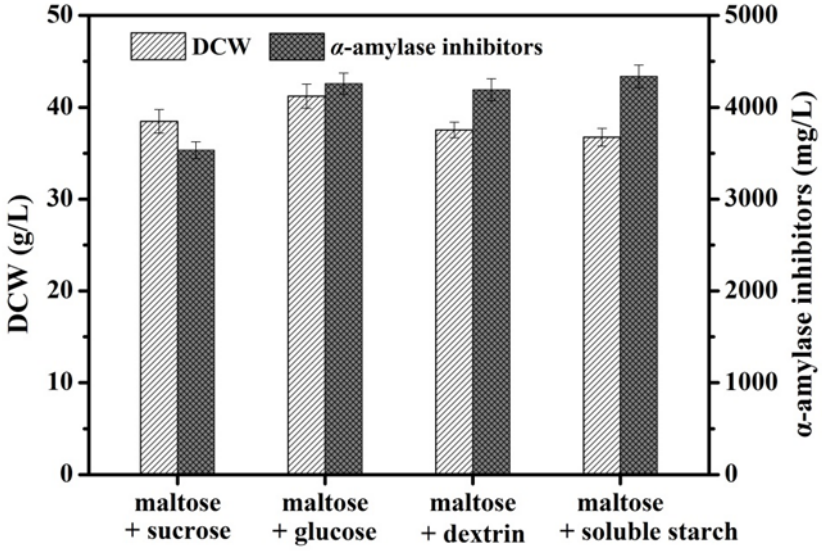

Fig.5 Effects of mixed carbon sources in feeding medium on *α*-amylase inhibitors production

The dry cell weight and the yield of *α*-amylase inhibitors in different mixed carbon source are plotted in Fig.5. With composite carbon source composed of maltose and soluble starch, *S.coelicoflavus* ZG0656 exhibited a relatively high weight of *α*-amylase inhibitors produced, the *α*-amylase inhibitors yield reached the maximum value of 4336 ± 23.45 mg/L, whereas with other mixed carbon source, the yield of *α*-amylase inhibitors is relatively lower comparing to the former. As mentioned before, maltose is not only used as the carbon source for fermentation processes but also acts as a precursor for *α*-amylase inhibitors biosynthesis. After maltose was exhausted, soluble starch was hydrolyzed by *α*-glucosidic hydrolase and consumed dramatically, accompanied by the rapid accumulation of *α*-amylase inhibitors.

Effect of the ratio of maltose and soluble starch in feeding medium on *α*-amylase inhibitors production

According to the biosynthetic pathway of *α*-amylase inhibitors, maltose and soluble starch directly or indirectly incorporated into *α*-amylase inhibitors [21]. Therefore, the ration of maltose and soluble starch in fermentation broths probably had an important role in *α*-amylase inhibitors biosynthesis. To explore the appropriate ratio of maltose and soluble starch for *α*-amylase inhibitors production in *S.coelicoflavus* ZG0656, the feeding medium was consisted of seven various mass ratios of maltose and soluble starch (3:1, 2:1, 1:1, 1:2, 1:3, 0:1 and 1:0, respectively). During the fed-batch fermentation process in 5-L jar fermenter, 60 g of total sugar/L of the above feeding medium was fed at 72, 96 and 120 h, respectively. The experimental results are listed in Table 2.

Table 2 Effects of the ratio of maltose and soluble starch in feeding medium on *α*-amylase inhibitors production

M:SS[a]	DCW (g/L)	*α*-amylase inhibitors (mg/L)
3:01	38.39 ± 0.21	4276 ± 21.33
2:01	38.01 ± 0.33	4517 ± 30.64
1:01	37.53 ± 0.24	4336 ± 23.45
1:02	37.28 ± 0.50	4210 ± 17.43
1:03	36.93 ± 0.28	4043 ± 24.17
0:01	36.76 ± 0.11	3950 ± 24.47
1:00	38.93 ± 0.10	4136 ± 14.97

[a] *Mass ratio of maltose and soluble starch in the feeding medium*

As shown in Table 2, the ratio of maltose and soluble starch had a significant effect on the *α*-amylase inhibitors. In the case of solely using maltose or soluble starch as the carbon source in feeding medium, only 4136 ± 14.97 and 3950 ± 24.47 mg/L of *α*-amylase inhibitors were obtained. When a 2:1 ratio of maltose and soluble starch was fed to the fermentation broth, the maximum *α*-amylase inhibitors (4517 ± 30.64 mg/L) was achieved. Therefore, based on the results and their multiple comparisons, it could be concluded that the feeding medium with a 2:1 ratio of maltose and soluble starch was favorable for *α*-amylase inhibitors biosynthesis in *S.coelicoflavus* ZG0656.

Effects of carbon source concentration in feeding medium on *α*-amylase inhibitors production

Because the osmolality was crucial to acarbose biosynthesis [22-23] and the osmolality could be performed by adjusting the concentration of medium components, the correlation of carbon source concentration with acarbose production was an attractive proposition. To explore the optimal total sugar concentration for *α*-amylase inhibitors production, the total sugar of broth was controlled at 40-50, 50-60, 60-70, 70-80, 80-90 and 90-100 g/L during the *α*-amylase inhibitors fermentation in the 5-L jar fermenter, respectively, by continuously feeding a 2:1 ratio of maltose and soluble starch to the fermentation broths. According to the results presented in Table 3, the *α*-amylase inhibitors production was strongly affected by the concentration of total sugar in the fermentation broth. When the total sugar concentration in broth was increased from 40-50 to 70-80 g/L, the *α*-amylase inhibitors yield and broth osmolality also gradually improved. Under 70-80 g/L of total sugar, a maximum *α*-amylase inhibitors yield of 4726 ± 18.78 mg/L was obtained. When the total sugar concentration was further increased to 90-100 g/L, the final *α*-amylase inhibitors production were significantly decreased to 4088 ± 15.69 g/L.

Table 3 Effect of carbon source concentration in feeding medium on α-amylase inhibitors production

Total sugar(g/L)	DCW(g/L)	*α*-amylase inhibitors (mg/L)
40-50	33.11 ± 0.12	4195 ± 24.37
50-60	37.19 ± 0.24	4407 ± 21.61
60-70	38.01 ± 0.33	4517 ± 30.64
70-80	39.59 ± 0.31	4726 ± 18.78
80-90	36.37 ± 0.16	4273 ± 12.33
90-100	31.27 ± 0.25	4088 ± 15.69

CONCLUSION

In this study, the optimal initial carbon source containing 3:1 of maltose and glucose and its optimal concentration was determined to be 100 g/L, and the feeding medium containing 2:1 of maltose and soluble starch and its optimal concentration was 70-80 g/L. Under the optimal conditions, a final *α*-amylase inhibitors concentration of 4726 mg/L was achieved after 168 h.

Acknowledgments

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