ISSN 1330-9862
(FTB-1513)

original scientific paper

Improvement of Fructanohydrolase Production in *Aspergillus niger* SL-09 by Sucrose Ester

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Received: May 18, 2005
Accepted: September 27, 2005

Summary

The influence of carbon sources, nitrogen sources, and the addition of sucrose ester on fructanohydrolase production was investigated. The enzyme production varied depending on the carbon source. Apart from that, it was found that the enzyme activities formed by *Aspergillus niger* SL-09 were enhanced dramatically by the addition of sucrose ester S-770 to the medium. The effect of sucrose ester on enzyme production was also studied on molecular level, and it was confirmed that the transcription was activated by the addition of sucrose ester to the medium. The response surface methodology (RSM) was used to optimize the composition for the production of fructanohydrolase, and the enzyme activities were enhanced more than 7-fold than those obtained in the basal medium.

Key words: *Aspergillus niger*, fructanohydrolase, sucrose ester, optimization

Introduction

Inulin source, such as Jerusalem artichoke, chicory, or dahlia, which can grow well on poor land and show high tolerance to frost and various plant diseases, have recently received much interest as renewable raw material for the production of fructose syrup (D-fructose) or other chemicals such as ethanol (*1*,*2*). Inulin is hydrolysed by two types of inulinases: exoinulinase (β-D-fructan fructanohydrolase, E.C. 3.2.1.80) and endoinulinase (2,1-β-D-fructan fructanohydrolase, E.C. 3.2.1.7). Exoinulinase catalyzes the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose) by cleaving the glycosidic linkages in polymer moiety. Exoinulinase were distinguishable from endoinulinase by their ability to hydrolyze sucrose (*3*). Thus, microbial fructanohydrolase plays an important role in the hydrolysis of inulin for its commercial exploitation.

A number of fungal, yeast, and bacterial strains have been used for the production of fructanohydrolase (*4*). Among them, *A. niger* is widely used (*5*), and much work has been done to enhance the activities of fructanohydrolases from this fungus, such as mutagenesis (*6*), optimization of the culture condition (*5*), identifying the genes which are coding for the fructanohydrolase (*7*) as well as carrying out x-ray crystallography on the isolated and purified protein (*8*), among other. It was reported that fructanohydrolase production by fungal culture was inducible (*9*), therefore, the current challenge is to further enhance the enzyme activities via metabolic engineering. In the present study, the effect of the addition of sucrose ester to the liquid culture of *A. niger* SL-09 was investigated, and RSM, an efficient experimental strategy, was used to optimize the media composition for increasing the fructanohydrolase production.

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Materials and Methods

Microorganisms and media

A. niger SL-09, an active producer of fructanohydrolase, which was used in carrying out the primary experiments, was isolated from the soil. This strain was grown on potato agar (*10*) slants at 30 °C for 3 days and then stored at 4 °C until use. *A. oryzae* 3045 and *A. ficuum* 2258 were obtained from the China Science Department. These strains were maintained on potato agar slants and subcultured every month. The basal medium used for the production of fructanohydrolase contained (in g/L): sucrose 20.0, peptone 20, $NH_4H_2PO_4$ 12.0, NaCl 5.0, $MgSO_4 \cdot 7H_2O$ 0.5, and $FeSO_4 \cdot 7H_2O$ 0.01, adjusted to pH=6.0 with 0.1 M of HCl or NaOH.

Production of fructanohydrolase in the media containing various surfactants

To determine the effect of the addition of various surfactants to the culture medium on the enhanced enzyme activities, Span 20 (sorbitan monolaurate, EEC No. 493), Span 40 (sorbitan monopalmitate, EEC No. 495), Span 60 (sorbitan monostearate, EEC No. 491), Span 80 (sorbitan monooleate, EEC No. 494) and four kinds of sucrose ester (sucrose ester of fatty acids, EEC No. E 473) (*11*): S-1570, S-970, S-770 and S-370 were added to the basal medium at the same concentration of 5 g/L, respectively, and the eight were compared as a culture medium for the production of fructanohydrolase. All of the cultures in this and the following tests were conducted in 250-mL shake flasks containing 50 mL of liquid medium at 30 °C for 5 days on a rotary shaker (140 rpm).

Fructanohydrolase production by A. niger SL-09 in medium containing sucrose ester S-770

In order to determine the effect of the addition of sucrose ester to the medium on the production of fructanohydrolase using *A. niger* SL-09, sucrose ester S-770 was added to the basal medium in the concentration of 5 g/L, and the medium without sucrose ester was used as a control. During the process, mycelia were periodically withdrawn and centrifuged at 5000 rpm and 4 °C for 5 min. Supernatant was used for the analysis of the activities of extracellular fructanohydrolases. To measure the activities of intracellular fructanohydrolases, the fungus mycelia were washed twice with phosphate buffer (0.05 M, pH=5.4), broken up by sonication (20 000 Hz, 10 min) in chilled water, and clear supernatant was obtained by centrifugation (10 000 rpm, 15 min).

In order to investigate whether the addition of sucrose ester to the medium was essential for the enhanced enzyme activities of fructanohydrolases, the sucrose ester was added to the basal medium at different times (at the beginning, and in 12-hour intervals).

Regulation of fructanohydrolase synthesis in A. niger SL-09

To determine whether the regulation of fructanohydrolase synthesis in *A. niger* SL-09 occurs at transcription or translation level, actinomycin (a repressor of transcription) and cycloheximide (a repressor of translation) (*12*) were added to the basal and sucrose ester-containing media after 48 h of fermentation with the concentration of 100 and 50 μg/mL, respectively, after which the samples were periodically withdrawn and used for the analysis of the enzyme activities.

Effect of additional nutrients on fructanohydrolase production

The effects of various additional nutrients (carbon source, nitrogen source, and mineral salt solution) on fructanohydrolase production were studied by adding these to the basal medium. Various carbon sources, including glucose, sucrose, inulin, and soluble starch, were added at the concentration of 30 g/L to the basal medium. Nitrogen sources including peptone, yeast extract, corn steep liquor, soybean flour, urea, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, and NH_4Cl were added to the basal medium to give a concentration of 30 g/L. To study the effect of mineral salt solution on fructanohydrolase production in the medium, 5 mL of salt solution was added to the basal medium. The mineral salt solution comprised (in g/L): K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 5.0, $CaCl_2 \cdot 2H_2O$ 1.0, $FeSO_4 \cdot 7H_2O$ 0.05, and $ZnSO_4 \cdot 7H_2O$ 0.05.

Optimization of selected nutrients using RSM

RSM was used to optimize the concentration of the three effective nutrients (sucrose ester, sucrose, and peptone), which resulted from the above studies. The lowest and highest concentrations of the selected ingredients in the media were: sucrose ester 2 and 6 g/L, sucrose 20 and 60 g/L, peptone 20 and 60 g/L, respectively.

Analytical methods

The extracellular inulinase (I) and invertase activities (S) were assayed by measuring the reducing sugar released from inulin and sucrose, respectively, as described by Pessoni *et al.* (*13*). One unit (U) of enzyme activity was defined as the amount of enzyme (in 1 mL), which liberates 1 μmol of fructose equivalent from inulin (inulinase) or sucrose (invertase) per min. Reducing sugars were estimated with 3,5-dinitrosalicylic acid (DNS), using fructose as standard (*14*). Total reducing sugar was assayed by the same method after acid hydrolysis (adjusted to pH=1.0 with sulfuric acid and 100 °C for 30 min). The pH was measured by pH-meter. Biomass concentration was determined by harvesting the mycelia pellets by filtration and freeze-drying them to a constant mass, while the dry mass was expressed as gram per liter of the fungal culture.

Results and Discussion

Effect of various surfactants on fructanohydrolase production

As shown in Fig. 1, for all the surfactants tested, the inulinase activity increased most significantly when sucrose ester S-770 was added to the medium. However, when the surfactant (Span), which was distinguished from sucrose ester by its sorbitol residues, was added to the basal medium, the enzyme activities obtained were

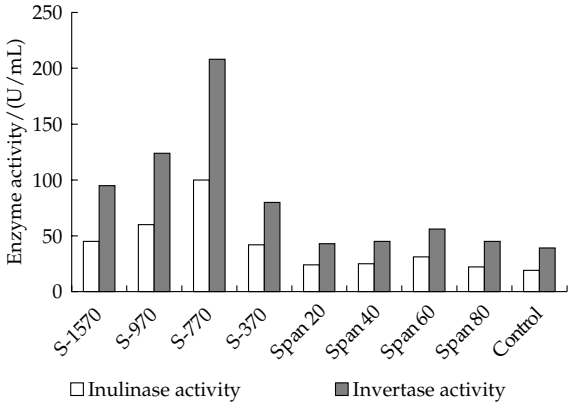

Fig. 1. Production of fructanohydrolase in the medium containing various Spans and sucrose esters with the same concentration of 5 g/L by *Aspergillus niger* SL-09. The fermentations were conducted in 250-mL shake flasks containing 50 mL of liquid medium at 30 °C for 5 days on a rotary shaker (140 rpm)

all lower than those in the medium containing sucrose ester but higher than those of the basal media. Those observations lead to the conclusion that sucrose residues of the sucrose ester molecule act as an active center for improving fructanohydrolase production.

Effect of sucrose ester S-770 on enzyme activities

When sucrose ester S-770 was added to the basal medium, the extracellular enzyme activities increased from 19.4 and 38.8 U/mL in the basal medium to 107 and 222 U/mL for inulinase and invertase, respectively. Likewise, the intracellular enzyme activities of inulinase and invertase were enhanced from 20.2 and 43.5 U/mL to 118 and 237 U/mL, respectively. Both extracellular and intracellular enzyme activities were enhanced 5-fold more than those in the basal medium after 5 days of submerged culture (Table 1). The addition of sucrose ester to the medium has very little effect on the cell growth. These observations lead to the conclusion that fructanohydrolase is an inducible enzyme, rather than a constitutive enzyme, which is similar to a previous report (*9*).

Table 1. Comparative fermentation parameters of *Aspergillus niger* SL-09 in the basal and the medium containing sucrose ester

Media	γ(biomass)/(g/L)	Extracellular enzyme activities/(U/mL)		Intracellular enzyme activities/(U/mL)	
		I*	S**	I*	S**
Basal media	20	19	38	20	43
Media containing sucrose ester	21	107	222	118	237

*I-inulinase, **S-invertase

As shown in Fig. 2, only in case of sucrose ester supplementation at the beginning and after 24 h of the culture, enzyme activities of more than 100 U/mL for inulinase and nearly 200 U/mL for invertase were obtained. The supplementation of sucrose ester at 48 h increased inulinase and invertase activities to 75 and 142 U/mL, respectively, but after 72 h it had very little effect on fructanohydrolase production. These observations indicated that the action of sucrose ester on fructanohydrolase production was almost complete within 72 h.

Effect of sucrose ester on the regulation of fructanohydrolase synthesis in A. niger SL-09

With the addition of actinomycin D, fructanohydrolase biosynthesis in the basal medium was reduced dra-

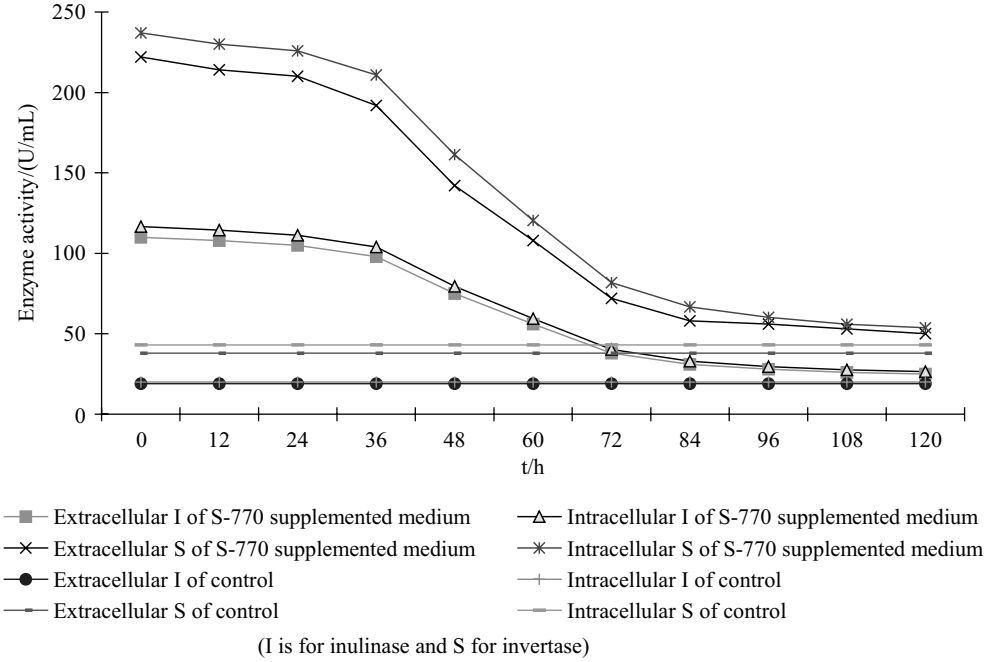

Fig. 2. Effect of sucrose ester S-770 added at each stage (at the beginning and then in 12-hour intervals) of culture on fructanohydrolase production

matically, and the synthesis of the enzyme nearly ceased. However, in the medium containing sucrose ester, the fructanohydrolase biosynthesis continued up to 10 h after the addition of actinomycin D, and the inulinase activity was increased up to 6 U/mL. This observation suggested that the amount of mRNA available for translation in the system of the medium containing sucrose ester was higher than that in the system of basal medium. When cycloheximide was added instead of actinomycin D, fructanohydrolase production decreased markedly in both basal and sucrose ester containing media.

The above observation suggested that the regulation of fructanohydrolase synthesis in *A. niger* SL-09 occurs at both transcriptional and translational level, and the enzyme synthesis was provoked by the addition of sucrose ester at transcriptional level.

Effect of sucrose ester on fructanohydrolase production by other Aspergillus strains

After 5 days of submerged culture, inulinase activity of 11.1 U/mL and invertase activity of 26.8 U/mL were produced by *A. oryzae* 3045 in the basal medium, while in the medium containing sucrose ester the enzyme activities increased more than 3-fold than those in the basal medium, which were 35.1 and 72.2 U/mL, respectively. Likewise, the enzyme activities of inulinase and invertase produced by *A. ficuum* 2258 were enhanced from 7.7 and 11.1 U/mL to 15.7 and 27.7 U/mL, respectively. From these results, it can be concluded that using different strains, the final enzyme activities were enhanced to different degrees by sucrose ester. These differences might be contributed to the different physiological properties of each strain.

Effect of the additional nutrients on fructanohydrolase production

As presented in Fig. 3, the inulin showed maximum enzyme yield, followed by sucrose. However, as sucrose was cheaper and locally abundantly available, it should be chosen as carbon source for inulinase production.

Among the nitrogen sources, peptone was the best, followed by yeast extract and soybean (Fig. 4). These results show that all inorganic nitrogen sources chosen in these experiments were not favourable for enzyme production. Studies also indicated that enzyme activities

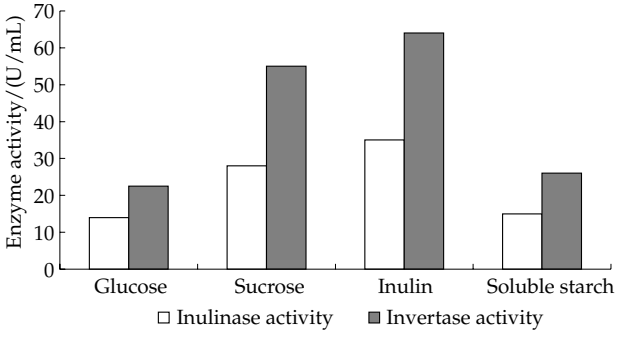

Fig. 3. Effect of carbon sources on fructanohydrolase production by *Aspergillus niger* SL-09. All the experiments were run in the media with various carbon sources of the same concentration of 30 g/L, at 30 °C for 5 days

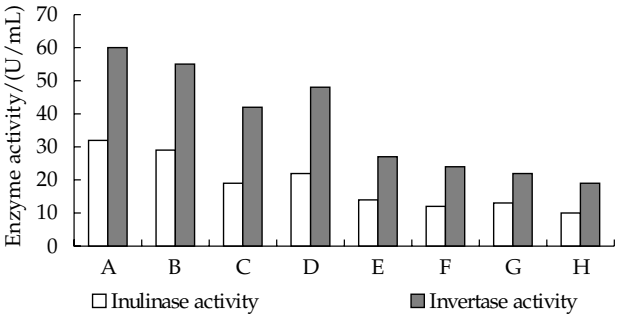

Fig. 4. Effect of nitrogen sources on fructanohydrolase production by *Aspergillus niger* SL-09. A, peptone; B, yeast extract; C, corn steep liquor; D, soybean flour; E, $NH_4H_2PO_4$; F, $(NH_4)_2SO_4$; G, NH_4Cl; H, urea. The nitrogen sources were added to the media with the same concentration of 30 g/L

were not enhanced significantly by the addition of mineral salt solution (data not presented). Therefore, among all the supplementary nutrients, sucrose ester, sucrose and peptone were found to be superior for higher enzyme production. Subsequently, experiments were conducted for optimization of these selected nutrients employing RSM.

Optimization of medium ingredients for fructanohydrolase production using RSM

The average inulinase activity obtained after 5 days of fermentation in 20 experiments of the chosen experimental design and results are shown in Tables 2 and 3. The factorial analysis of variance indicated that the concentration of sucrose ester (X_1), peptone (X_2), sucrose (X_3), and the second power of sucrose ester (X_1^2) all met the significant level of 0.15 of enzyme production by *A. niger* SL-09. A linear regression equation could be obtained from the regression results of fractional factorial experiment:

$$Y = 126.5009 + 27.34652\,X_1 + 18.2141\,X_2 + \\ + 27.39726\,X_3 - 31.48994\,X_1^2$$

where Y is the measured response of inulinase activity. The regression coefficients and determination coefficient (R^2) for the linear regression model of enzyme production are presented in Table 4. The model was highly significant ($p<0.001$) and $R^2=0.88$. From the above results, the economically optimized composition of fermentation medium was (in g/L): sucrose 60, peptone 40, sucrose ester 6, NaCl 5.0, $MgSO_4{\cdot}7H_2O$ 0.5, and $FeSO_4{\cdot}7H_2O$ 0.01. The maximum response predicted from the model was 150 U/mL. Repeated experiments were performed to verify the predicted optimum. The results from three replications (148, 152 and 146 U/mL for inulinase; 295,

Table 2. Range and levels of experimental variables

Factors	Level of factors		
	–1	0	1
Sugar ester (X_1/(g/L))	2	4	6
Peptone (X_2/(g/L))	20	40	60
Sucrose (X_3/(g/L))	20	40	60

Table 3. Experimental design and results of RSM

Run	X_1	X_2	X_3	Inulinase activity/ (U/mL)
1	0	–1	–1	57.2
2	0	–1	1	81.1
3	0	1	–1	83.2
4	0	1	1	142.1
5	–1	0	–1	77.1
6	–1	0	1	117.7
7	1	0	–1	115.7
8	1	0	1	142.1
9	–1	–1	–1	20.3
10	–1	1	1	75.1
11	1	–1	–1	85.2
12	1	1	1	162.4
13	0	0	0	107.6
14	0	0	0	117.7
15	0	0	0	154.2

Table 4. Regression results of RSM for inulinase production

Parameter[a]	Inulinase activity	
	Estimate	Pr>/T/
Intercept	126.5	<0.0001
X_1 (sucrose ester)	27.3	0.017
X_2 (peptone)	18.2	0.067
X_3 (sucrose)	27.3	0.0168
X_1^2(sucrose ester)	–31.4	0.0402

[a]all variables are significant at the 0.1500 level

303 and 292 U/mL for invertase) were coincident with the predicted value and the model was proven to be adequate.

Sucrose ester, composed of sucrose residue attached by stearic acid through an ester-type linkage, is a food additive and used as a surfactant to improve the solubility of ingredients in liquid (*15*). The molecule of sucrose ester is amphipathic, containing both hydrophilic sucrose and hydrophobic stearic acid residues. In aqueous solution, molecules of sucrose ester form themselves into a spherical micellar structure in which the stearic acid is hidden inside the micellar and the sucrose interacts with the surrounding water molecules (*16*). The surface of the lipid bilayers of the cell membranes is hydrophilic with polar headgroups; therefore, it is easy for sucrose residue to attach to the fungus cell membrane (*17*). Sucrose ester, containing sucrose residues, which attach to the surface of fungus cells, acts as a persistent signal to initiate a response in the cell to promote the transcription of mRNA available for fructanohydrolase synthesis, which was confirmed by our observations.

RSM is an efficient experiment strategy to seek optimal conditions for a multivariable system. It has been successfully employed for optimization of the medium composition and operating conditions in many bioprocesses (*18*). Inulinase and invertase activities were enhanced to 150 and 298 U/mL after 5 days of culture using optimized medium, over 7 times higher than those in the basal medium. The price of sucrose ester is about 4500 $/t, therefore, the enzyme yield in the optimized medium was enhanced to about $3.8 \cdot 10^5$ U/$ for inulinase and $7.5 \cdot 10^5$ U/$ for invertase, respectively, which was nearly 5 times higher than that obtained in the basal medium (about $8 \cdot 10^4$ U/$ for inulinase and $16 \cdot 10^4$ U/$ for invertase).

Conclusion

As a result of the data presented in this work, sucrose ester was proved to be a very efficient activator for the transcription of mRNA available for fructanohydrolase production, especially for the strains of *Aspergillus*, as was the case with the strain of *A. niger* SL-09 under study. The sucrose ester was first used as a significant factor to optimize the composition for fructanohydrolase production, and the final extracellular and intracellular enzyme activities in the optimized medium of *A. niger* SL-09 were enhanced more than 7-fold than those in the basal medium Therefore, to further enhance enzyme activities of fructanohydrolases in the liquid culture, sucrose ester, as a significant factor, should be taken into account.

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Poboljšanje proizvodnje fruktanohidrolaze dodatkom estera šećera pri uzgoju *Aspergillus niger* SL-09

Sažetak

Ispitan je utjecaj izvora ugljika i dušika te dodatak estera šećera na proizvodnju fruktanohidrolaze s pomoću *Aspergillus niger* SL-09. Proizvodnja enzima mijenjala se ovisno o izvoru ugljika, ali se enzimska aktivnost dramatično povećala dodavanjem estera šećera S-770 (ester masne kiseline) u podlogu. Učinak estera šećera na proizvodnju enzima ispitan je na molekularnoj razini, pa je utvrđeno da on aktivira transkripciju. Sastav podloge za proizvodnju fruktanohidrolaze optimiran je metodom odzivnih površina, a enzimska se aktivnost povećala više od 7 puta u usporedbi s proizvodnjom na osnovnoj podlozi.