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Production of β-Fructofuranosidase from *Aspergillus niger* ATCC 20611

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ABSTRACT

An increase in magnesium sulfate decreased the ratio of extracellular/intracellular β-fructofuranosidase activity in shaking flask cultivation of *Aspergillus niger* ATCC 20611. At 0.4% $MgSO_4$ concentration, the enzyme ratio was decreased to 54%, but the total enzyme activity was increased 1.12 times more than that with no salt addition. Three ammonium salts tested, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, and NH_4NO_3 could not substitute yeast extract as a nitrogen source because of decreasing in the specific growth rate, enzyme yield and productivity. In this study, yeast extract with vitamin solution gave the highest values of specific growth rate, enzyme yield and productivity. Based on the fermentation kinetic study in aerobic batch culture under controlled pH and temperature, the specific growth rate, enzyme yield and productivity were improved to 0.0414 h^{-1}, 65.16×10^3 U/g sucrose and 1.89×10^3 U/g cell h, respectively. These values were increased 1.85, 10.5 and 497.9 times, respectively, comparing with shaken flask culture.

Key words : β-Fructofuranosidase, *Aspergillus niger* ATCC 20611, fermentation kinetics

INTRODUCTION

Oligosaccharides are important in sweetener industry because of their wide applications, such as low calorie sweetener for diabetics and functional food for enhancing growth of intestinal microorganisms (Oku *et al*., 1984; Hidaka *et al*., 1986; Yun *et al*., 1994). These well-known oligosaccharides are cyclodextrins (Hara *et al*., 1994), isomalto-oligosaccharides (Kohmoto *et al*., 1991), soybean-oligosaccharides (Wada *et al*., 1992) and fructo-oligosaccharides (Hidaka *et al*., 1988; Jung *et al*., 1993; Yun and Song, 1993).

Among them, fructo-oligosaccharides play an important role for health food and their processing are readily industrialized as an alternative sugar substitute in food industry. The production is enzymatically derived process with the help of β-fructofuranosidase (EC 3.2.1.26) produced from *Aspergillus niger* ATCC 20611 (Hidaka *et al*., 1988), and *Aureobasidium pullulans* KFCC 10524 (Jung *et al*., 1987, 1993; Yun and Song, 1993; Yun *et al*., 1994). Fructo-oligosaccharides produced from sucrose are commercially available in 55–60% maximum content and the remaining is a mixture of glucose, fructose and sucrose.

Aspergillus niger ATCC 20611 was a promising strain for the production of β-fructofuranosidase (Hidaka *et al*., 1988). When sucrose was used as substrate, its conversion by β-

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fructofuranosidase formed a mixture of 1^F(1-β-fructofuranosyl)$_n$-sucrose based fructo-oligosaccharides, i.e., 1-kestose (n=1, GF_2), nystose (n=2, GF_3) and fructofuranosyl nystose (n=3, GF_4). As reported by Burnett and Trinci (1979), magnesium ion plays an important role for synthesizing the fungal cell wall. This may affect on the formation of β-fructofuranosidase both intra- and extracellularly. Moreover, magnesium salt gave an excellent result in producing fructosyltransferase by *Aureobasidium pullulans* KFCC 10245 (Jung *et al*., 1987). Therefore, in this work, an effect of magnesium sulfate was studied on the production of intra- and extracellular β-fructofuranosidase from *Aspergillus niger* ATCC 20611. Moreover, three ammonium salts i.e. $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3 were investigated as substituting nitrogen source for an expensive yeast extract. The fermentation kinetics describing this fungal growth and β-fructofuranosidase production was also conducted in a laboratory 2–l fermenter.

MATERIALS AND METHODS

Microorganism *Aspergillus niger* ATCC 20611 was purchased from American Type Culture Collection, Rockville, Maryland, USA. The stock culture was prepared by growing on potato dextrose agar slant at 30°C for 3–4 days and kept at 4–8°C in refrigerator. The culture was transferred using the same procedure every 2–4 weeks.

Chemicals All chemicals used in the experiments, e.g. sucrose, $MgSO_4$, yeast extract and ammonium salts were laboratory-grade reagents.

β-fructofuranosidase production The studies on effects of $MgSO_4$ and ammonium salts for enzyme production were conducted by shaken flask culture. Its optimization was also studied in a 2–l laboratory fermenter.

(1) Shaken flask culture The 3–4 days old culture was inoculated into a 250-ml Erlenmeyer flask containing 25 ml Hidaka medium (2.0% sucrose, 1.2% yeast extract and 0.2% carboxymethyl cellulose (CMC) in 0.3 M McIlvaine buffer pH 5.0) (Hidaka *et al*., 1988) and incubated in an incubator shaker at 28°C rotating at 280 rpm for 24 h. The culture was then transferred to a 1–l Erlenmeyer flask containing 250 ml Hidaka medium without CMC. The culture condition in incubator shaker was maintained at 28°C rotating at 280 rpm. Samples were withdrawn every 3–6 h for determination of cell and sucrose concentrations, β-fructofuranosidase activity and/or nitrogen sources concentration.

Different amount of $MgSO_4$ was added as follows; 0, 0.1, 0.2, 0.3 and 0.4% in Hidaka medium containing 1.0% sucrose and 0.5% yeast extract. To replace yeast extract, three ammonium salts, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3 were used in Hidaka medium containing 2.5% sucrose and 0.2% $MgSO_4$ with or without 1.0% additional vitamin solution (0.01 g each per 100 ml of distilled water: biotin, pyridoxine-HCl, thiamine-HCl, riboflavin, p-aminobenzoic acid and nicotinic acid) (Atkinson and Mavituna, 1983).

As the nitrogen content of yeast extract, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3 were 8.4, 21.20, 21.22 and 17.50%, respectively. These necessary amounts of nitrogen source were calculated in 20% excess according to 5.21% nitrogen content of *Aspergillus niger* and its biomass yield of 0.41 g g^{-1}. Therefore, the final concentrations of yeast extract, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3 were 0.76, 0.30, 0.30 and 0.37%, respectively.

(2) Fermenter culture The inoculums were prepared using two consecutive shaken flask cultures in Hidaka medium (2.0% sucrose, 1.2% yeast extract, 0.2% carboxymethyl cellulose and 0.2% $MgSO_4$ in 0.3 M McIlvaine buffer pH 5.0); 15 ml in 250-ml Erlenmeyer flask, transferred to a

500-ml Erlenmeyer flask containing 150 ml Hidaka medium and controlled at 28°C rotating at 280 rpm for 24 h. This culture was inoculated into a 2-l Mini Jarfermenter (M-100, Tokyo Rikakikai Co., Ltd, Japan) with 1.5 l of Hidaka medium containing 2.5% sucrose, 1.5% yeast extract, 0.2% $MgSO_4$ and 0.1% antifoam in 0.3 M McIlvaine buffer at pH 5.0. The cultivation was controlled at pH 5.0 with addition of 2.0 N NaOH or 2.0 M H_3PO_4, 28°C, 600 rpm agitation and 1 vvm aeration. Samples were taken every 1.5–3 h of culture time for determining the concentrations of cell, sucrose and nitrogen and β-fructofuranosidase activity.

Analytical methods Cell concentration was determined by cell dry weight, drying at 105°C for 24 h. Sucrose concentration was determined by Phenol-H_2SO_4 method (Dobois *et al*., 1956). The Weatherburn method for ammonia determination was used for analyzing nitrogen concentration using $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3 as standards (0–40 μg/ml) (Weatherburn, 1967). An organic nitrogen contained in yeast extract was determined by Ninhydrin method using yeast extract as standard (0-200 mg/ml) (Moore and Stein, 1954).

Enzyme activity The culture filtrate was obtained by filtration and its enzyme activity was determined as extracellular β-fructofuranosidase. The cells retained after filtration and washed with distilled water was extracted by freezing and thawing twice. After filtration, the supernatant was determined as intracellular β-fructofuranosidase. The reaction mixture (1.0 ml crude extract, 1.0 ml 0.1 M McIlvaine buffer pH 5.0 and 1.0 ml 25% sucrose solution) was carried out at 40°C for 10 minutes and terminated with DNS addition followed by boiling at 100°C for 5 minutes (Bernfeld, 1955). The reducing sugar was subsequently analyzed by using 3,5-dinitrosalicylic acid (Miler, 1959). One unit of β-fructofuranosidase was defined as 1 μmole of glucose liberated in 1 minute under specified condition.

RESULTS AND DISCUSSION

Aspergillus niger ATCC 20611 was previously reported as a promising strain for the production of β-fructofuranosidase because of its high enzyme productivity (Hidaka *et al*., 1988). Therefore, the strain was selected for studying the production of β-fructofuranosidase (β-FFase) in this work.

Effect of $MgSO_4$ concentrations on β-fructofuranosidase production

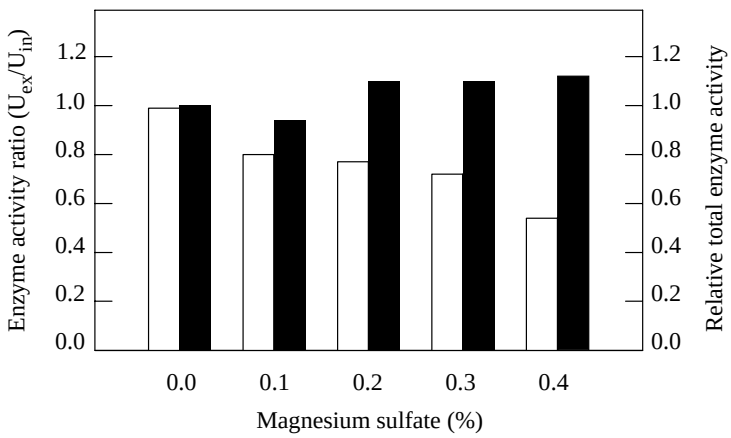
$MgSO_4$ concentrations were varied from 0–0.4% for studying the production of intra- and extracellular β-FFase from *Aspergillus niger* ATCC 20611. From experimental result, an increase of $MgSO_4$ decreased the ratio of extra- to intracellular β-FFase (U_{ex}/U_{in}), in which the ratio of U_{ex}/U_{in} was reduced to 0.54 at the highest concentration of $MgSO_4$ (0.4%). On the other hand, the total activity of β-FFase was increased 1.12 times at 0.4% $MgSO_4$ (Figure 1). This supports the report that magnesium ion accelerates the activities of chitin synthase and glucan synthase regulating the fungal cell wall synthesis (Burnett and Trinci, 1979). As a result, synthesizing cell wall might retard the excretion of intracellular enzyme due to limited permeability. Therefore, more intracellular and less extracellular β-FFase were obtained, resulting an decrease of U_{ex}/U_{in}. However, the reason for $MgSO_4$ affecting an increase of total β-FFase activity was still not clear. Similarly, Jung *et al*. (1987) have reported that $MgSO_4$ was able to increase an intracellular enzyme activity of *Aureobasidium pullulans* fructosyltransferase by 140% without affecting on its total activity, when increasing its concentration from 0.05 to 0.2%. While the $MgSO_4$ concentration beyond 0.2% (0.2-0.4%), intracellular enzyme activity was not changed.

In conclusion, the least concentration of $MgSO_4$ with the highest ratio of U_{ex}/U_{in} must be

considered as an optimal $MgSO_4$ concentration. However, 0.2% $MgSO_4$ was selected as an optimal concentration for cultivating *Aspergillus niger* ATCC 20611 in the following experiments, since this concentration can improve 1.10 times total β-FFase activity with relatively high U_{ex}/U_{in} (Figure 1). Therefore, an enzyme preparation from *Aspergillus niger* ATCC 20611 must be exploited by the whole cell extraction for obtaining both intra- and extracellular β-FFase as the total production of β–fructofuranosidase.

Effect of ammonium salts on β-fructofuranosidase production

To replace an expensive yeast extract, three ammonium salts, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3 have been examined for the growth of *Aspergillus niger* ATCC 20611 and its total β-FFase production. Unfortunately, the specific growth rate, enzyme yield and productivity were not improved by addition of these ammonium salts (Figure 2). Maximal values of growth (0.0224 h^{-1}), β-FFase yield (6.19×10^3 U/g sucrose) and



$MgSO_4$ (%)	Enzyme activity	
	U_{ex}/U_{in}	Relative total activity
0	0.99	1.00
0.1	0.80	0.94
0.2	0.77	1.10
0.3	0.72	1.10
0.4	0.54	1.12

Note: The 1–1 flask culture of 250 ml medium containing 1.0% sucrose and 0.5% yeast extract with varied $MgSO_4$ concentrations was carried out on incubator shaker at 280 rpm and 30°C. The samples were taken at 4 h of culture time for enzyme determination.

Figure 1 Effect of magnesium sulfate on extracellular/intracellular and relative total enzyme activity of β-fructofuranosidase by *Aspergillus niger* ATCC 20611.
□ Uex/Uin ■ Relative total enzyme activity

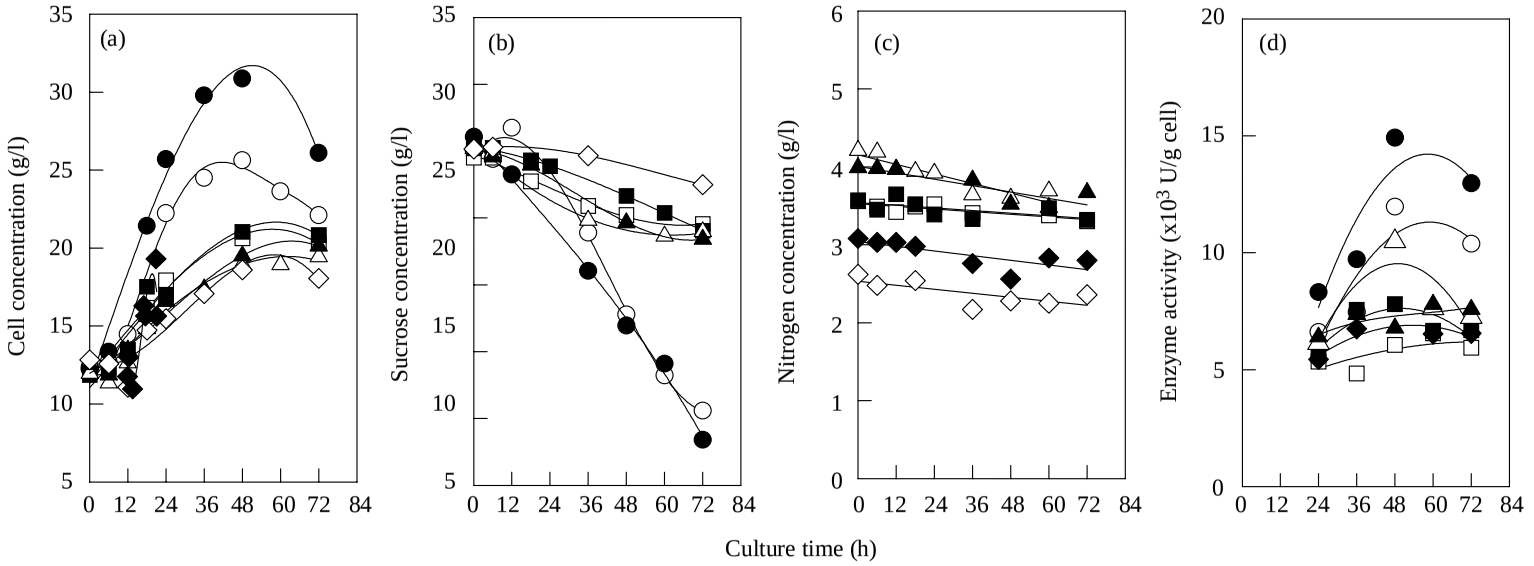


Figure 2 Comparison of nitrogen sources for growth and β-fructofuranosidase production of *Aspergillus niger* ATCC 20611 by shaking culture at 28°C, 1 vvm and 600 rpm for 3 days.
(a) Fungal growth (b) Sucrose utilization (c) Nitrogen utilization (d) Enzyme production
Symbols : ○ Yeast extract ● Yeast extract + vitamins □ $(NH_4)_2SO_4$ ■ $(NH_4)_2SO_4$+ vitamins
△ $(NH_4)_2HPO_4$ ▲ $(NH_4)_2HPO_4$+vitamins ◇ NH_4NO_3 ◆ NH_4NO_3+vitamins

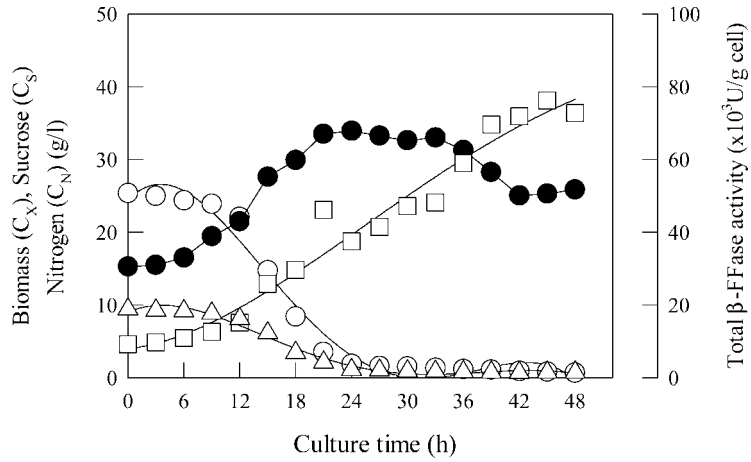


Figure 3 Aerobic batch culture of *Aspergillus niger* ATCC 20611 in 2.0–1 fermenter containing 1.5 1 medium with 2.5 % sucrose, 1.5% yeast extract and 0.2% $MgSO_4$ controlled at pH 5.0, 1 vvm, 600 rpm and 28°C for 2 days.
symbols : ● Biomass ○ Sucrose
△ Nitrogen □ Total β-FFase activity

productivity (3.8 U/g cell h) were obtained by using yeast extract and vitamin solution. In all cases, with supplementation of vitamin, fungal growth and enzyme productivity were increased (Table 1). This indicated that the vitamin solution was not able to replace the growth factors contained in yeast extract. But the replacement of yeast extract by vitamin slightly enhanced the fungal growth and enzyme productivity. As a result, yeast extract clearly showed its better suitability as nitrogen source containing growth factors for the production of β–fructofuranosidase.

Among ammonium salts with vitamin added, $(NH_4)_2HPO_4$ gave the maximal specific growth rate (0.0095 h^{-1}) and β-FFase productivity (1.4 U/g cell h), but enzyme yield (0.57×10^3 U/g sucrose) was minimal. While NH_4NO_3 gave the highest β-FFase yield (6.10×10^3 U/g sucrose) and similar enzyme productivity (1.3 U/g cell h) (Table 1). Except for yeast extract with or without vitamin, thus, the resulting synthetic medium might be formulated with the addition of $(NH_4)_2HPO_4$ and vitamin according to the highest specific growth rate and enzyme productivity obtained. But this medium formulation was not appropriate for the enzyme yield. Nevertheless, as the merit of yeast extract, Hidaka medium containing yeast extract without additional vitamins was still used in the following fermenter culture for the production of β-fructofuranosidase.

The production of β-fructofuranosidase in fermenter

Under controlled aerobic condition in 2-l fermenter (pH 5.0, 28°C, 1 vvm aeration and 600 rpm agitation), fermentation kinetics was verified for the growth of *Aspergillus niger* ATCC 20611 and essentially for the production of β-fructofuranosidase in Hidaka medium containing yeast extract without additional vitamins. Comparing with the shaken flask culture using yeast extract and vitamin, *Aspergillus niger* ATCC

Table 1 Effect of nitrogen sources on growth and enzyme production of *Aspergillus niger* ATCC 20611 by shaken culture with medium containing 2.5% sucrose and 0.2% $MgSO_4$ (initial pH 5.0) at 28°C and 280 rpm for 3 days.

Parameters	Yeast extract		$(NH_4)_2SO_4$		$(NH_4)_2HPO_4$		NH_4NO_3	
	Without vitamin	With vitamin	Without vitamin	With vitamin	Without vitamin	With vitamin	Without vitamin	With vitamin
μ	0.0176	0.0224	0.0073	0.0079	0.0075	0.0095	0.0057	0.0060
q_S	15.8	16.4	4.2	5.2	5.5	5.9	2.4	3.7
q_P	3.6	3.8	0.6	1.2	1.3	1.4	1.2	1.3
q_N	ND	ND	2.2	2.1	1.8	2.7	2.2	2.5
$Y_{X/S}$	0.55	0.71	0.32	0.33	0.45	0.57	0.30	0.36
$Y_{P/S}$	3.88	6.19	1.51	0.57	0.56	0.57	2.09	6.10
$Y_{X/N}$	ND	ND	10.15	14.45	12.08	17.40	6.62	9.23

Note : ND = not determine
Specific rates ; μ (h^{-1}), q_S (mg sucrose/g cell h), q_P (U/g cell h), q_N (mg nitrogen/g cell h)
Yields ; $Y_{X/S}$ (g cell/g sucrose), $Y_{P/S}$ ($x10^3$U/g sucrose), $Y_{X/N}$ (g cell/g nitrogen)

Table 2 Kinetic parameters obtained from aerobic batch culture of *Aspergillus niger* ATCC 20611 containing 2.5% sucrose, 1.5% yeast extract and 0.2% $MgSO_4$ at controlled conditions of pH 5.0, 28°C, 1 vvm aeration and 600 rpm agitation.

Conditions	μ (h^{-1})	q_S (mg/g h)	q_P ($x10^3$U/g h)	q_N (mg/g h)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ ($x10^3$U/g)	$Y_{X/N}$ (g/g)
Complex medium (pH 5.0)	0.0414	36.3	1.89	25.6	0.53	65.16	0.96

Note : Specific growth rate (μ), specific rates of sucrose consumption (q_S), enzyme production (q_P) and nitrogen consumption (q_N)
Biomass yields based on substrate ($Y_{X/S}$) and nitrogen ($Y_{X/N}$) and product yield based on sucrose ($Y_{P/S}$)

20611 cultivated in fermenter found its higher specific growth rate of 0.0414 h^{-1}, 10.5 times higher β-FFase yield (65.16×10^3 U/g sucrose) and 497.9 times higher β-FFase productivity (1.89×10^3 U/g cell h) (Table 2). This shows very clearly an enhancement of fungal growth and enzyme production in fermenter culture under controlled conditions.

CONCLUSION

β-fructofuranosidase from *Aspergillus niger* ATCC 20611 was found to be both intra- and extracellular enzyme. An increase of magnesium sulfate decreased the ratio of U_{ex}/U_{in}, but potentially increased the total activity of β-fructofuranosidase. An optimal concentration of magnesium sulfate was decided as 0.2% in this work for enhancing the

production of β-fructofuranosidase. Although $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3 were not suitable for replacing yeast extract, $(NH_4)_2HPO_4$ or NH_4NO_3 might be relatively potential as nitrogen source. Fermentation kinetic study on β-fructofuranosidase production in fermenter culture showed remarkably an improvement of growth, enzyme yield and productivity. Comparing with shaken flask culture, approximately 500 times higher in β-fructofuranosidase productivity was obtained. However, further study on optimization would possibly enhance enzyme yield and productivity, such as pH and temperature. Optimal pH and temperature affecting fungal growth and the formation of β-fructofuranosidase may be totally different. For designing a production process of β-fructofuranosidase, these unseen factors are very much necessary.

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