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Production of β-Fructofuranosidase by *Arthrobacter* sp. and Its Application in the Modification of Stevioside and Rebaudioside A

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Summary

Arthrobacter sp. 10137 has been used to produce β-fructofuranosidase (FFase). Sucrose and corn steep powder in an optimized ratio of 10:1 were the best carbon and nitrogen sources for enzyme production in a shake flask. The maximum FFase activity was 26.69 U/mL after 22.5 h in batch culture, and the crude FFase, obtained by ultrafiltration and $(NH_4)_2SO_4$ fractionation, was purified about 7-fold as measured by specific activity from the crude culture filtrate. The FFase was specific for introduction of a fructose molecule at the C_{19} position on both the stevioside and rebaudioside A, with high transfructosylating activity of 65 % after 15 h of incubation.

Key words: β-fructofuranosidase, purification, stevioside and rebaudioside A, structure modification

Introduction

β-Fructofuranosidase (EC 3.2.1.26; FFase) catalyzes not only the hydrolysis of nonreducing termini of various β-D-fructofuranoside substrates to release β-fructose, but also the transfructosylation of various substrates that have a hydroxyl group (acceptor) with sucrose (donor). FFase have been used to commercially produce fructooligosaccharides (FOS) such as kestose (GF2), nystose (GF3), and 1^F-β-fructofuranosylnystose (GF4), which are popular functional foods owing to their prebiotic properties (*1*,*2*). Nowadays, there is a growing effort to apply FFase as a biocatalyst to synthesize more glycosides with useful functional groups by effective enzymatic process which is simple and cheap compared to a complicated chemical one (*3*,*4*). FFase are also potential industrial enzymes for the production of different chemicals that are not easily synthesized by chemical means (*5*,*6*).

FFase enzymes isolated from fungi, yeast, and/or bacteria have shown little promise in industrial applications due to their differences in transfructosylating activity. The activity and characteristics of FFase from different organisms are shown in Table 1 (*7-12*).

Steviosides are a mixture of nine diterpenoid glucosides isolated from *Stevia rebaudiana* in China, with stevioside (St) and rebaudioside A (RA) being the main components (Scheme 1, Table 2). St and RA are low-calorie, safe, and natural sweeteners with high sweetness and pharmacological activity (*13*) and they have great commercial potential. Unfortunately, they have a bitter aftertaste, which affects their qualitative characteristics. Regarding the structural and functional relationships of St and RA it has been reported that glycosyl substitution at C_{13} and C_{19} of stevioside had a close relationship with the bitterness and sweetness, respectively (*14*). Derivates produced by linking a fructosyl either through its C_2 or

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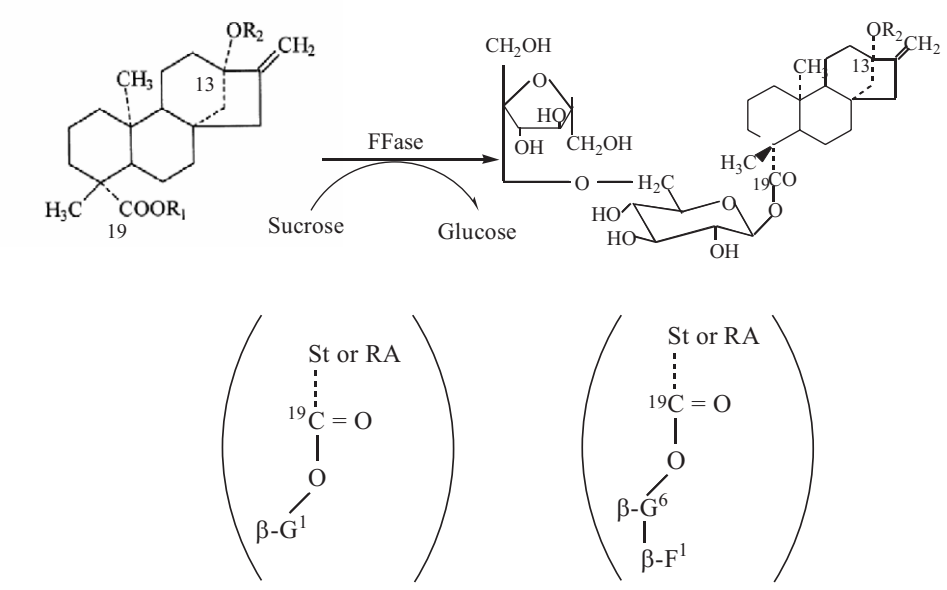
Table 1. FFase activity and characteristics of different organisms

Microorganism	FFase assay	FFase acitivity	Substrate specificity	References
E. coli: gene from *Bifidobacterium lactis* DSM10140^T	Hydrolytic activity	Different enzyme activity was shown according to different substrate	High affinity to terminal β(2–1) glycosyl linkages between fructose moieties	*7*
Aureobasidium pullulans DSM2404	Hydrolytic and transfructosylase activity	Ratio of transfructosylase activity to hydrolytic activity by FFase I-V is 14.3, 12.1, 11.7, 1.28 and 8.11.	n.d.	*8*
Aureobasidium pullulans DSM2404	Hydrolytic activity	The total crude FFase I activity was 195 U/g dry mass cells. FFase (II-V) activity was not described	n.d.	*9*
Aspergillus niger IMI303386	Transfructosylase activity	Transfructosylase activity is 2.4 U/mL in shake flask culture	n.d.	*10*
Aspergillus japonicus TIT-90076	Hydrolytic and transfructosylase activity	Transfructosylase activity is 660 U/mL under optimal culture conditions, and hydrolytic activity is very low	n.d.	*11*
Schwanniomyces occidentalis	Hydrolytic and transfructosylase activity	Transfructosylase activity is 0.4 U/mL in a 1-litre batch culture	Broad substrate specificity on hydrolyzing sucrose, 1-kestose, nystose and raffinose with different catalytic efficiencies	*12*

*no biocatalysis on St and RA was researched; n.d. not determined



Scheme 1. Enzymatic modification of steviosides by FFase. Refer to Table 2 for R_1 and R_2

C_6 hydroxyl group to stevioside or rebaudioside to give a C_{19}-O-β-fructosyl derivative showed improved sweet taste (*15*,*16*). Thus, attention has been focused on improvement of the taste quality, and an enzyme modification of their structure was regarded as a good way to solve this problem (*13*,*17*).

In the present study, *Arthrobacter* sp. 10137 was cultured in shake flasks and 5-litre fermentors (batch mode) under optimized conditions to produce crude FFase that was isolated by the concentration with ultrafiltration and $(NH_4)_2SO_4$ fractionation. This crude FFase was used to modify the stevioside and rebaudioside A, giving good overall yield of 65 %, specifically at the C_{19} position (Scheme 1).

Materials and Methods

Microorganism culture

Arthrobacter sp. 10137 was maintained on slant plate. The medium contained (in %): beef extract 1, peptone 0.5, NaCl 0.5, and agar 2, pH=7.2. Seed cultures were prepared by inoculating single colonies into 40 mL (in 250-mL flasks) of this culture medium containing no

Table 2. Major glycoside components and characteristics of *Stevia rebaudiana* leaves

Diterpenoid glucoside	R_1	R_2	Content fraction/%	Relative sweetening power (with respect to sucrose)	References
Stevioside	β-G	-β-G^2-β-G^1	55–65	250–300	*4*
Steviolbioside	H	-β-G^2-β-G^1	<0.05	100–125	*5*
Rebaudioside A	β-G	-β-G^2-β-G^1 | ^{3} β-G^1	22–28	350–450	*5*
Rebaudioside B	H	-β-G^2-β-G^1 | ^{3} β-G^1	0.08–0.5	300–350	*5*
Rebaudioside C	β-G	-β-G^2-α-R^1 | ^{3} β-G^1	4–7	50–120	*6*
Rebaudioside D	β-G^2-β-G^1	-β-G^2-β-G^1 | ^{3} β-G^1	0.3–0.8	200–300	*7*
Rebaudioside E	β-G^2-β-G^1	-β-G^2-β-G^1	<0.05	250–300	*7*
Dulcoside	β-G	β-G^2-α-R^1	0.8–1.6	50–120	*8*
Rebaudioside F	β-G	β-G^2-β-X^1 | ^{3} β-G^1	n.d.	n.d.	*9*

G glucose, R rhamnose, X xylose, H hydrogen atom; n.d. not determined

agar. The inocula were grown at 30 °C in an orbital shaker at 250 rpm. After 20 h of cultivation, 2.5 mL of the seed culture were used to inoculate 50 mL of production medium in a 500-mL glass flask, and this culture was grown under the same conditions as the seed culture. The composition of production medium included glucose, sucrose, yeast extract, corn steep powder, $MgSO_4 \cdot 7H_2O$, $NaNO_3$ and $(NH_4)_2HPO_4$, with the amount of each component varying for different experimental processes. The selected carbon sources were glucose and sucrose, and the selected nitrogen sources included yeast extract, corn steep liquor and $NaNO_3$.

Batch fermentation

Fermentation was carried out in a 5-litre New Brunswick fermentor equipped to monitor and control temperature, pH, agitation, aeration and dissolved oxygen. The culture was inoculated with 5 % (by volume) of the inoculum and then cultivated at 30 °C. During the course of cultivation, the dissolved oxygen tension was adjusted to above 20 % by regulation of the rate of agitation and aeration. The composition of production medium for batch fermentation included (in %): sucrose 4, corn steep powder 4, $MgSO_4 \cdot 7H_2O$ 0.13 and $(NH_4)_2HPO_4$ 0.4. The corn steep powder was dissolved in water and sterilized independently, while the other media were mixed and sterilized in the fermentor. Prior to the addition of inoculum, the sterilized corn steep powder solution was poured into the fermentor. Cell density was measured at 600 nm using distilled water as a blank.

Enzyme preparation

All procedures were done at 4 °C. The supernatant was clarified by centrifugation (12 000×*g*, 20 min, 4 °C), filtered through a 0.45-μm cellulose acetate filter and then concentrated by ultrafiltration through a 10-kDa relative molecular mass cut-off membrane (Omega 10 K, Centrasette II, Pall, USA). The crude enzyme solution was concentrated by ammonium sulphate precipitation (40–90 %) and the precipitated fractions were collected by centrifugation (12 000×*g*, 30 min). Each fraction was dissolved in phosphate buffer (50 mM, pH=6.5, 50 mL) and the crude fractionated enzyme was lyophilized and kept at 4 °C for further application by analysis and catalysis.

β-fructofuranosidase activity assay

The β-fructofuranosidase (FFase) activity was determined by measuring the reducing sugars released by the hydrolysis of high concentration of sucrose (*18*). One unit of FFase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute under the described conditions. Crude enzyme solution (0.5 mL) and 0.5 mL of 40 % (*m*/*V*) sucrose (in 50 mM phosphate buffer, pH=6.5) were mixed at 30 °C in an orbital shaker at 200 rpm. After 10 min, the reaction was stopped by boiling in a water bath for 10 min. Blanks were used with a heat-inactivated enzyme sample (100 °C, 10 min). The mixture was treated with 3,5-dinitrosalicylic acid (DNS) reagent (2.0 mL), boiled in a water bath for 2 min, cooled to ambient temperature, and then diluted to 25 mL with distilled water. The absorbance (*A*) was read at 540 nm and the amount of glucose was determined from a standard curve prepared under identical conditions.

Protein assay and electrophoretic analysis

Protein concentration was assayed by a protein assay kit (Bio-Rad, USA) with bovine immunoglobulin G

as a standard. SDS-PAGE was carried out on 10 % polyacrylamide slab gels, and was stained with Coomassie Brilliant Blue R-250.

Enzymatic modification of stevioside and rebaudioside A

The steviosides were provided by Bodun Company (Shenzhen, PR China), and the content of St and RA was 55 and 28 %, respectively. The transfructosylation reaction was performed in potassium phosphate buffer (20 mL, 50 mM, pH=6.5) containing 9 mM stevioside or 4 mM rebaudioside A, 1.0 M sucrose, and 303 U of crude FFase. The reaction system was incubated at 30 °Ñ for 20 h at 200 rpm, and the samples were withdrawn at intervals, and analyzed by HPLC.

HPLC analysis of reaction products

Stevioside (St), rebaudioside A (RA) and their product derivatives were determined by HPLC equipped with a UV detector (213 nm) using a Sun FireTM C18 column (5 μm, 4.6×150 mm, Waters, USA) with the mobile phase being acetonitrile/water=30:70 at a flow rate of 0.5 mL/min. The conversion of stevioside and rebaudioside A was calculated with the following equations:

Conversion of stevioside: (St-F)/(St+St-F)·100 /1/

Conversion of rebaudioside A: (RA-F)/(RA+RA-F)·100 /2/

where St-F is converted stevioside, St is unconverted stevioside, RA-F is converted rebaudioside A, and RA is unconverted rebaudioside A.

Results and Discussion

Effect of carbon and nitrogen sources on cell growth and FFase production

Since sucrose and glucose are readily available and cheap carbon sources, they were selected in our experiments to explore their effect on the cell growth and production of FFase. Each carbon source was added to the basal medium at 40 g/L. As shown in Fig. 1, glucose was a good carbon source for the cell growth (cell density here and in Figs. 2 and 3 was calculated as A_{600nm}× dilution in arbitrary units, a.u.), while it was not good for the production of FFase. In contrast, FFase production was higher when sucrose was used as the carbon source although the cell biomass was lower. It was noted that good cell growth may not be a determining factor for high production of FFase by *Arthrobacter* sp. 10137. Thus, sucrose was chosen as the carbon source for use in the following experiments. The highest FFase activity was 4.2 U/mL at 24 h of cultivation.

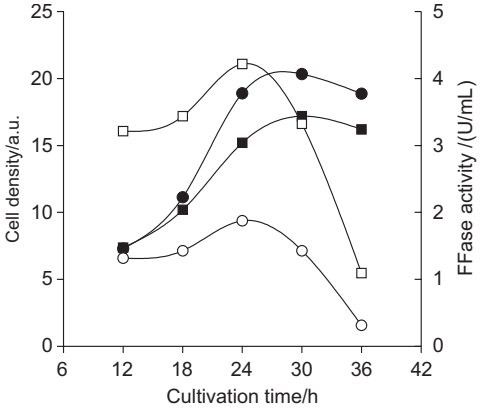


Fig. 1. Effect of carbon sources on the cell growth and production of FFase by *Arthrobacter* sp. 10137. Cell growth curve: ■ sucrose, ● glucose; enzyme production curve: □ sucrose, ○ glucose

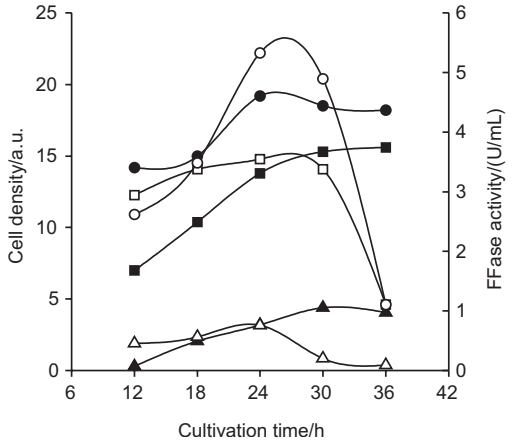


Fig. 2. Effect of nitrogen sources on the cell growth and production of FFase by *Arthrobacter* sp. 10137. Cell growth curve: ■ yeast extract, ● corn steep liquor, ▲ $NaNO_3$; enzyme production curve: □ yeast extract, ○ corn steep liquor, △ $NaNO_3$

The effect of nitrogen sources on the cell growth and FFase production was also studied and the results are shown in Fig. 2. The selected nitrogen sources included yeast extract, corn steep powder and $NaNO_3$, and an equal molar concentration of nitrogen for each nitrogen source was added to the culture media. The highest cell biomass and FFase production were achieved when corn steep liquor was used as a nitrogen source, while the cell density (at A_{600}) and FFase activity increased respectively to 18.9 and 5.2 U/mL after 24 h of cultivation. $NaNO_3$, which is an inorganic nitrogen source, and yeast extract gave relatively lower cell growth and FFase activity, so corn steep liquor, a by-product of corn

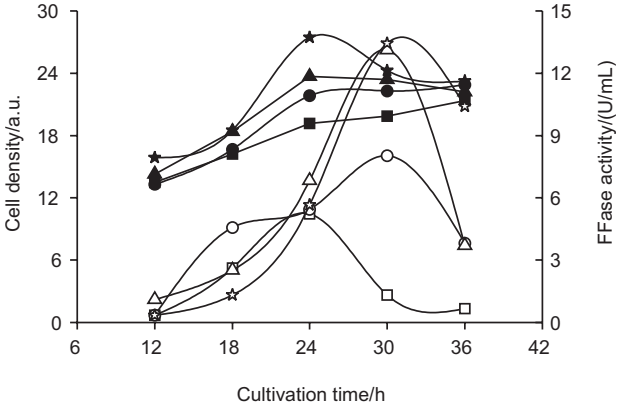


Fig. 3. Effect of the ratio of carbon to nitrogen sources on the cell growth and the production of FFase by *Arthrobacter* sp. 10137. Cell growth curve: ■ C:N=16:1, ● C:N=13:1, ▲ C:N=10:1, ★ C:N=7:1; enzyme production curve: □ C:N=16:1, ○ C:N=13:1, △ C:N=10:1, ☆ C:N=7:1

starch processing, was used as the nitrogen source to produce FFase in the later experiments.

The molar ratio of nitrogen and carbon sources is a major factor that affected cell growth and enzyme production by microorganisms. While the sucrose was kept constant at 4 % in the culture media, the concentration of corn steep powder was altered to obtain molar ratios of C:N of 16:1, 13:1, 10:1, or 7:1. The effects of various ratios on the cell growth and FFase production are shown in Fig. 3. Cell biomass increased with the increase of nitrogen (C:N ratio decreasing), while the FFase production increased less when the ratio of carbon to nitrogen changed from 10:1 to 7:1, with the FFase activity of 13.1 and 13.4 U/mL, respectively. Corn steep powder is a mixture containing many ingredients, and too high concentration of it in the medium leads to low solubility and intense colour. Thus, on an economical scale C:N of 10:1 was optimal in the shake flask experiments. When C:N was 10:1, the cell density and FFase activity were 23.7 and 13.1 U/mL, respectively.

Time profiles of batch fermentation in the fermentor

Fig. 4 shows typical time profiles of the cell growth and the FFase production by *Arthrobacter* sp. 10137 in a 5-litre fermentor under the above optimal culture conditions without pH control. Cell density was in the lag phase for the first 15 h of cultivation, and rose significantly after this to achieve a maximum of 68.1 at 26 h. FFase production followed the same trend over the same period with the maximum FFase acitivity of 26.69 U/mL being reached at the cultivation time of 22.5 h. It was also noted that pH was related to FFase production since with the pH decrease between 15 and 22.5 h of cultivation, FFase production increased. An indepth study of the effect of pH on FFase production will be reported later.

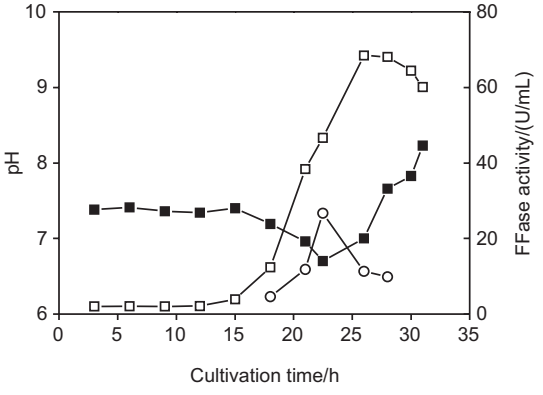


Fig. 4. The time profiles of cell growth and FFase production in a 5-litre fermentor without pH control. ■ pH, ○ FFase, □ cell density

FFase activity

Relative FFase activity in the precipite, which is FFase activity in the precipitate divided by total FFase activity, increased with the increment of mass fraction of $(NH_4)_2SO_4$ (Fig. 5), with the maximum protein precipitation obtained when the mass fraction of $(NH_4)_2SO_4$ was 80 % (Fig. 6). The precipitate, which was collected at different mass fractions of $(NH_4)_2SO_4$, was dissolved in the deionized water, and after analysis by SDS-PAGE, it could be seen that the optimal mass fraction of $(NH_4)_2SO_4$ was 80 %.

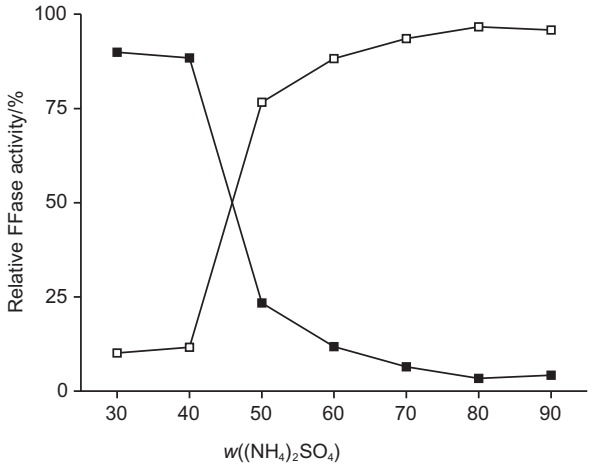


Fig. 5. Effect of $(NH_4)_2SO_4$ concentration on the relative FFase activity in the supernatant and precipitate. ■ Relative FFase activity in the supernatant, □ relative FFase activity in the precipitate

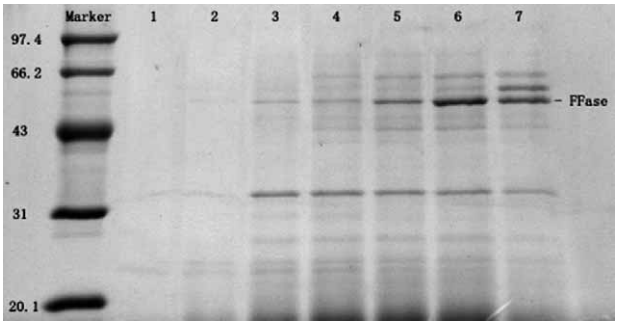


Fig. 6. SDS-PAGE of the crude FFase in the precipitate at different mass fractions of $(NH_4)_2SO_4$. The molecular mass of FFase was about 55.8 kDa. Lanes 1–7 present the mass fractions of $(NH_4)_2SO_4$: 30, 40, 50, 60, 70, 80 and 90 %, respectively

A volume of 3 L of crude culture filtrate was concentrated by using ultrafiltration, and then $(NH_4)_2SO_4$ was added (80 %) to the concentrated supernatant. The results of the total process are summarized in Table 3. The fractionated enzyme exhibited 90 % of the total initial activity and there was about 7-fold increase in specific activity when compared with the crude culture filtrate.

Modification of stevioside and rebaudioside A

The crude FFase was used to catalyze the conversion of St and RA into fructosylated products (St-F and RA-F) (Scheme 1) and the products were analyzed by HPLC (Fig. 7). It could be seen that the FFase from *Arthrobacter* sp. 10137 was specific for the C_{19} position on the St and RA, which is important for the removal of bitterness from steviosides. It was also noted that the crude FFase showed the same high transfructosylating activity (65 %) after 15 h on both St and RA (Fig. 8).

Table 3. Summary of the purification of FFase from *Arthrobacter* sp. 10137

Step	*m*(total protein)	Total activity	Specific activity	Purification	Yield
	mg	U	U/mg	fold	%
Crude culture filtrate	267711.90	78884	0.29	1.00	100.00
Concentrated supernatant after ultrafiltration through 10-kDa membrane	69214.68	78847	1.14	4.51	99.95
Salting-out by $(NH_4)_2SO_4$	34606.15	70996	2.05	7.07	90.00

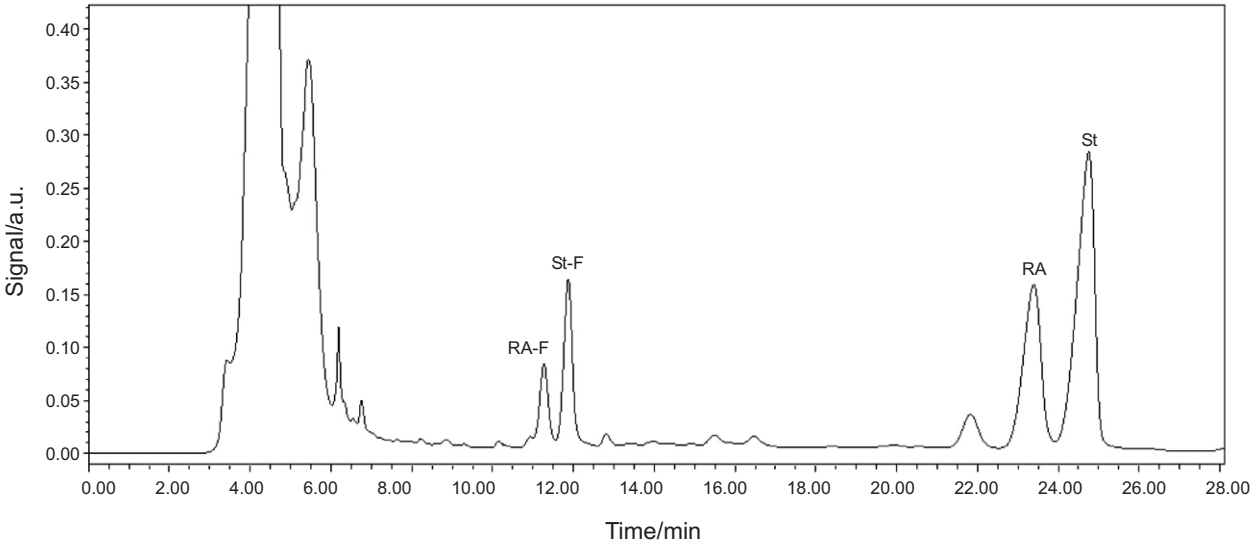


Fig. 7. HPLC analysis of the reaction substrate and products

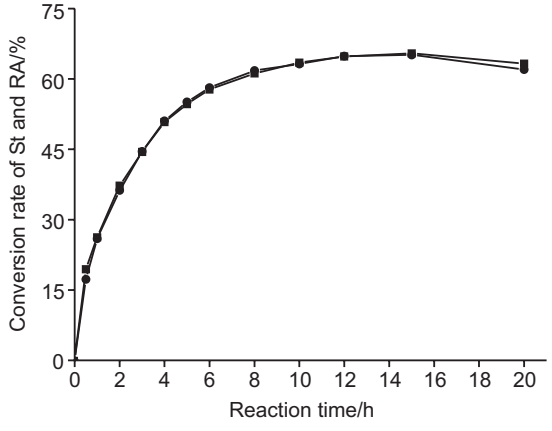


Fig. 8. Time profile of the conversion ratio of stevioside (■) and rebaudioside A (●)

Conclusions

A simple method is reported here for cultivation of *Arthrobacter*, extraction and concentration of FFase at high yield allowing the introduction of a fructose molecule specifically at the C_{19} position of St and RA. Therefore, it is a good enzyme to be used to modify St and RA and improve their taste. This research will facilitate industrial applications and further investigation is in progress. Sucrose and corn steep powder in an optimized ratio of 10:1 were regarded as the best carbon and nitrogen sources for enzyme production in a shake flask. The maximum FFase activity was 26.69 U/mL after 22.5 h in a batch culture. Ultrafiltration and $(NH_4)_2SO_4$ salting-out were adequate to concentrate the enzyme at 90 % of the total initial activity when compared with the crude culture filtrate. The maximum conversion ratio of stevioside and rebaudioside A was 65 % after 15 h.

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