ORIGINAL RESEARCH PAPER

Simple and efficient enzymatic transglycosylation of stevioside by *β*-cyclodextrin glucanotransferase from *Bacillus firmus*

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Abstract Stevioside was subjected to 1,4-intermolecular transglycosylation using *β*-cyclodextrin glucanotransferase (*β*-CGtase) produced from an alkalophilic strain of *Bacillus firmus*. The reaction was carried out by traditional, ultrasound-assisted and microwave-assisted techniques. Reaction under microwave conditions was faster and was completed in 1 min yielding two 1,4 transglycosylated products, 4′-*O*-alpha-D-glycosyl stevioside (I) and 4″-*O*-alpha-D-maltosyl stevioside (II) in 66% and 24%, respectively. The optimum transglycosylation occurred by using stevioside (1.24 mmol), *β*-CD (1.76 mmol) and *β*-CGtase (2 U/g) under microwave assisted reaction (MAR) in 5 ml sodium phosphate buffer (pH 7) at 50°C and 80 W power. MAR is therefore potentially a useful and economical method for faster transglycosylation of stevioside.

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Introduction

Stevioside, an entkaurene diterpene glycoside, is a constituent (5–10%) in *Stevia rebaudiana* leaves and has therapeutic importance as substitute of sugar for diabetics. It is 250–300 times sweeter than sugar and has a low calorie value (Brandle et al. 1998). Its after-taste bitterness restricts its use for human consumption and limits its application in food and pharmaceutical products. To overcome this problem, attention has been paid for producing de-bittered stevioside by modifying it through biotransformations (Abelyan et al. 2004, Kochikyan et al. 2006, Fukunaga et al. 1989, Ishikawa et al. 1990, Lobov et al. 1991, Kitahata et al. 1989, Yamamoto et al. 1994). However, yields of these reaction products were very low and, in certain cases, nine complex products were formed and bitterness was removed partially.

CGTase is produced by various *Bacillus* spp. and used for transglycosylation of stevioside. These reactions have been carried out by traditional technique and take longer time durations to complete (Abelyan et al. 2004). Application of ultrasound and microwave irradiation for stimulating enzymatic reactions are known (Capello et al. 2004, Sharma

et al. 2007, Bachu et al. 2007) but have largely remained unexplored. To the best of our knowledge, enzymatic transglycosylation of stevioside under ultrasound and microwave-assisted irradiation has not been reported so far. The objective of the present study was to compare transglycosylation yields of stevioside under traditional, ultrasound and microwave-assisted methods using β-CGTase as a biocatalyst produced from *alkalophilic* strain of *Bacillus firmus* microorganism and β-cyclodextrin (β-CD) as substitute for glucose donor.

Materials and methods

Enzyme and chemicals

Stevioside was isolated in pure form from *Stevia rebaudiana* leaves, purified and characterized in our laboratory (see supplementary information). β-Cyclodextrin (β-CD) was from Sigma (USA). Dextrin was purchased from Merck (India). *Bacillus firmus* 5119 was provided by National Collection of Industrial Microorganism, Pune, India.

Enzyme preparation

CGTase was produced using optimized culture medium as described by Gawande and Patkar (1999) but instead of starch dextrin was used. Fermentations were carried out in 14 l New Brunswick 110 fermenter (New Brunswick scientific Company, New Jersey, U. S. A.) with 10 l working volume at 28°C and 0.5 vvm airflow rate. pH of the medium was maintained at 9 ± 0.1 with 1% (w/v) Na_2Co_3 and dissolved O_2 was maintained above 30% oxygen saturation using an automatic agitation control. Foam was controlled with automatic addition of silicon oil antifoam (Catrang, Hico products, Mumbai). Dry cell weight was estimated from the OD_{600}. After fermentation (see Fig. 1), the culture was cooled to 10°C, centrifuged at $13,200 \times g$ and the supernatant was filtered using a hollow fiber membrane (pore size = 0.1 μm). The enzyme in the permeate was concentrated using 10 kDa ultrafiltration membrane. Activity of β-CGTase was determined by phenolphthalein method (Goel and Nene 1995) and is represented in U/g. The amount of β-cyclodextrin produced was estimated from a standard curve. One
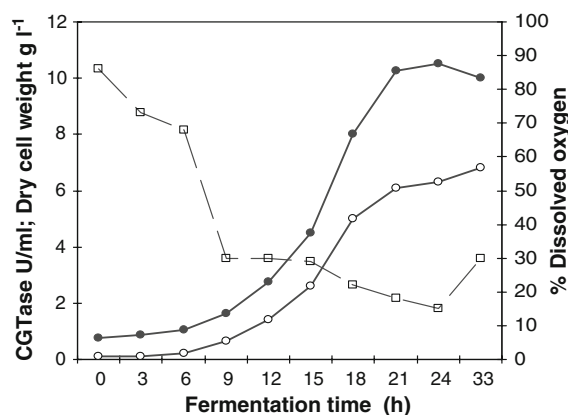

Fig. 1 Correlation of dissolved oxygen (%) and CGtase activity with fermentation time: % dissolved oxygen (*open square*), Dry cell weight g l^{-1} (*filled circle*) and CGTase production U/ml (*open circle*) by *Bacillus firmus*. For enzyme production: dextrin 40 g/l, yeast extract 10 g/l, peptone 10 g/l, $(NH_4)H_2PO_4$ 4 g/l, $MgSO_4$ $7H_2O$ 0.5 g/l and Na_2CO_3 10 g/l was autoclaved and inoculated with a loop

unit of β-CGTase is defined as the amount of enzyme required to produce 1 μmol β-CD/min.

Microwave-assisted reaction

Stevioside (1.24 mmol) and β-cyclodextrin (1.76 mmol) were dissolved in sodium phosphate buffer (5 ml) pH 7 at 50°C. To this β-CGTase (2 U/g) was added and the reaction mixture was held in a microwave oven (2,450 MHz, 300 W) at 80 W and 50°C for 1 min to carry out the reaction. The reaction was stopped by heating to 100°C for 10 min. The reaction was monitored by TLC using ethyl acetate/ethanol/H_2O (8:2:1.2, by vol) as solvent. The developed plate was dried and spots were visualized by spraying with acetic anhydride/sulfuric acid/ethanol (1:1:10, by vol, Jaitak et al. 2008). The reaction product after purification yielded 664 mg (I), 240 mg (II) with 90% total yield (Fig. 2). I and II were confirmed as 4'-*O*-alpha-D-glycosyl stevioside and 4''-*O*-alpha-D-maltosyl stevioside by ^1H, ^{13}C NMR, HMQC, HMBC and LCMS-MS spectra.

4'-*O*-alpha-D-glycosyl stevioside (I)

Rf = 0.50, ethyl acetate/ethanol/water (80:20:12, by vol), m.p 210–213°C. ^1H NMR (500 MHz, DMSO) anomeric protons 5.29 (1H, d, J = 7.2 Hz), 5.03 (1H, d, J = 3.4 Hz), 4.49 (1H, d, J = 7.6 Hz), 4.36 (1H, d,
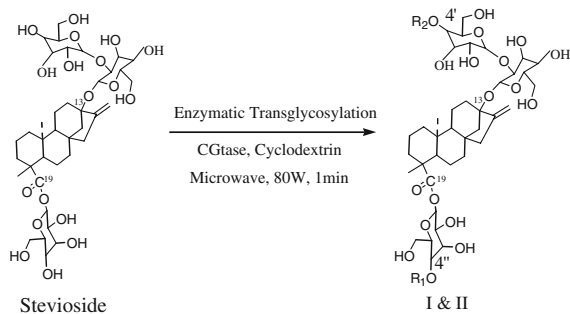
Fig. 2 Reaction scheme for transglycosylation of stevioside in MAR system. Reaction conditions: molar ratio of stevioside to β-CD 1:2, 2 U/g CGtase in 5 ml buffer at 80 W, 50°C for 1 min. $R_1 = H$, $R_2 = -\alpha$-G^4 (I, 4′-O-α-D-glycosyl stevioside); $R_1 = -\alpha$-$G'''^4\alpha$-G''''^4, $R_2 = H$ (II, 4″-O-α-D-maltosyl stevioside)

$J = 7.3$ Hz), ^{13}C NMR (125 MHz, DMSO) 176.1 (C-19), 154.1 (C-16), 105.1 (Glc-1′), 104.3 (C-17), 101.2 (Glc-1″), 96.8 (Glc-1), 94.6 (C-1‴), 84.9 (C-13), 83.1 (Glc-2), 79.6 (Glc-4′), 78.1 (Glc-5″, Glc-3‴), 77.5 (Glc-3″), 77.4 (Glc-5‴), 72.5 (Glc-2‴), 77.0 (Glc-5), 76.6 (Glc-3), 75.9 (Glc-5′), 75.8 (Glc-3′), 73.9 (Glc-2′), 73.7 (Glc-2‴), 70.7 (Glc-4‴), 70.4 (Glc-4″), 69.9 (Glc-4), 61.2 (Glc-6′), 61.0 (Glc-6′), 60.9 (Glc-6‴), 60.7 (Glc-6), 56.8 (C-5), 53.5 (C-9), 47.2 (C-15), 44.0 (C-14), 43.9 (C-4), 41.4 (C-7), 40.5 (C-1), 40.3 (C-10), 40.2 (C-8), 37.8 (C-3), 35.8 (C-12), 28.4 (C-18), 21.6 (C-6), 20.4 (C-11), 19.1 (C-2), 15.6 (C-20). TOF LC-MS (CH$_3$CN/H$_2$O, 0.1% formic acid) C$_{44}$H$_{71}$O$_{23}$ [M + H]$^+$ (calc) 967.89, [M + H]$^+$ (found) 967.76.

4″-O-alpha-D-maltosyl stevioside (II)

Rf = 0.40, ethyl acetate/ethanol/water (80:20:12, by vol), m.p. 221–223°C. ^{1}H NMR (500 MHz, DMSO) anomeric protons 5.30 (1H, d, J = 7.2 Hz), 5.02 (1H, d, J = 3.9 Hz), 5.01 (1H, d, J = 3.4 Hz), 4.53 (1H, d, J = 7.6 Hz), 4.39 (1H, d, J = 7.3 Hz), ^{13}C NMR (125 MHz, DMSO) 176.1 (C-19), 154.1 (C-16), 105.1 (Glc-1′), 104.3 (C-17), 101.4 (Glc-1‴), 101.2 (Glc-1‴), 96.8 (Glc-1), 94.4 (Glc-1″), 84.9 (C-13), 83.4 (Glc-2), 79.8 (Glc-4‴), 79.6 (Glc-4″) 77.3 (Glc-5‴), 77.0 (Glc-5, Glc-3‴), 76.6 (Glc-3), 75.9 (Glc-5′, Glc-5″), 75.8 (Glc-3′, Glc-3″), 75.4 (Glc-3‴, Glc-5‴), 73.9 (Glc-2‴), 73.7 (Glc-2′), 73.6 (Glc-2‴), 72.9 (Glc-2″), 71.1 (Glc-4‴), 70.6 (Glc-4′), 69.9 (Glc-4), 61.4 (Glc-6‴), 61.2 (Glc-6‴), 61.0 (Glc-6′), 60.9 (Glc-6″), 60.7 (Glc-6), 56.8 (C-5), 53.5 (C-9), 47.2

(C-15), 43.9 (C-4, C-14), 41.4 (C-7), 40.5 (C-1), 40.2 (C-8), 39.8 (C-10), 37.8 (C-3), 35.8 (C-12), 28.4 (C-18), 21.6 (C-6), 20.4 (C-11), 19.1 (C-2), 15.6 (C-20). TOF LC-MS (CH$_3$CN/H$_2$O, 0.1% formic acid) C$_{50}$H$_{81}$O$_{28}$ [Mr + H]$^+$ (calc) 1129.89 [Mr + H]$^+$ (found) 1129.74.

Statistical analysis

All experiments (except NMR spectra) were performed in triplicate. Data reported are mean ± standard deviation (SD).

Results and discussion

Transglycosylation is an important process for structural modification and derivatization of molecules for their better solubility and enhanced biological properties. It allows conversion of lipophilic compounds into hydrophilic ones, improves their pharmacokinetic properties and acts effectively in the body system. In the present work, transglycosylation of stevioside was made using traditional, ultrasound and microwave-assisted techniques for enhancing its sweetness and solubility. The results are given in Table 1.

Different parameters were selected to standardize microwave-assisted reaction (MAR) for optimization. The effect of variable power irradiation in microwave oven and time duration to complete the reaction are given in Fig. 3. Heating inside microwave oven is directly proportional to power irradiation and also depends on nature of the samples and solvents used. The decrease in yields of reaction products I and II above or below 80 W power is attributed either to overheating or inability to reach the desired conditions inside the microwave oven. Release of glucose moieties from the substrate (β-CD) for transglycosylation is dependents on availability of particular energy level inside the microwave oven. The reaction yield was observed maximum at 80 W as the desired power level in 1 min.

The selective concentration of β-CD, enzyme activity, effect of pH and temperature for optimisation of the reaction are given in Figs. 4 and 5. With gradual increase in concentration of β-CD upto 1.76 mM, equilibrium of the reaction favoured towards transglycosylation but further increase in concentration,

Table 1 Yield comparison of enzymatic reactions of stevioside by traditional, ultrasound and microwave-assisted methods
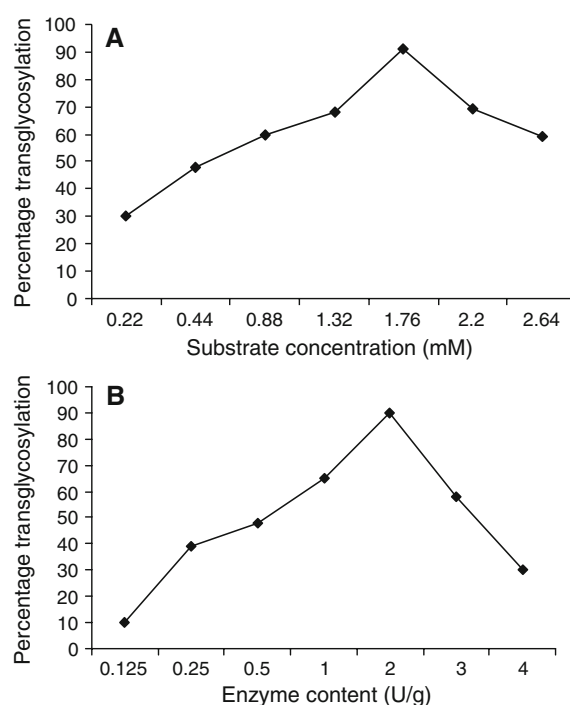
Reaction[a]	Time	Conditions	P-I (%) (n = 3)	P-II (%) (n = 3)	Total Yield (n = 3)
Traditional	12 h	50°C, 200 rpm	46 ± 0.4	24 ± 0.3	70 ± 0.5
Ultrasound assisted	10 min	Pulse length 2 s on, 1 s. off, duty 40%	50 ± 0.5	25 ± 0.7	75 ± 0.09
Microwave assisted	1 min	80 W at 50°C	66 ± 0.2	24 ± 0.4	90 ± 0.41

[a] Reaction conditions: Traditional method [stevioside (1.24 mmol), β-cyclodextrin (1.76 mmol), β-CGTase (2 U/g stevioside) in buffer (5 ml) at 50°C]; Ultrasound-assisted reaction [stevioside (1.24 mmol), β-cyclodextrin (1.76 mmol) in buffer (10 ml), β-CGTase (2 U/g) under sonication (20 KHz, 400 W, Pulse length 2 s on and 1 s. off, duty 40%) for 10 min]; Microwave-assisted reaction [stevioside (1.24 mmol), β-cyclodextrin (1.76 mmol), β-CGTase (2 U/g) in a microwave oven (2450 MHz, 300 W) at 80 W, 50°C for 1 min
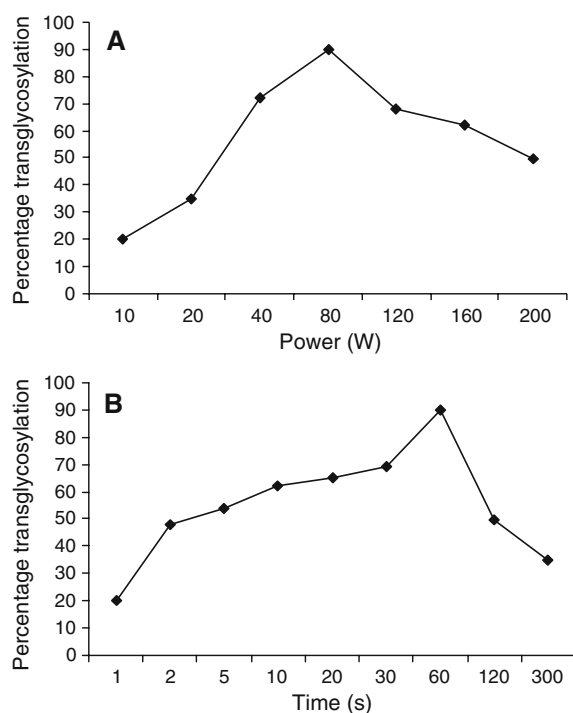
Fig. 3 Effect of power (W) and time (s) on transglycosylation of stevioside in the MAR system

Fig. 4 Effect of substrate and enzyme concentration on transglycosylation in the MAR system

decreased the rate of transglycosylation due to decrease in water activity of the system (a_w). Using enzyme activity in the range of 0.12–4 U/g, yields of I and II enhanced and reached maximum (90%) at 2 U/g. Beyond 2 U/g enzyme activity, there was substantial fall in the yields of I and II possibly because of structural instability of the enzyme.

Earlier, CGtase produced from mesophilic, halophilic and thermophilic strains of *Bacillus* species was used for catalyzing transglycosylation reaction with enzyme activity 8–9 U/g, 11–11.5 U/g and 15–16 U/g (Abelyan et al 2004). In the present investigation,

CGtase fron alkalophilic strain of *B. firmus* worked with low activity (2 U/g) and yielded transglycosylated products in higher yield. This shows that under microwave conditions CGtase when used in lower activity can transglycosylate with higher catalyzing efficiency as in traditional method where it works at a higher activity. The reaction favoured at pH 7 and decrease in yield of two products was observed under basic conditions because of the partial hydrolysis of additional attached glucose moieties due to the fall in enzyme activity. When reaction was carried out at
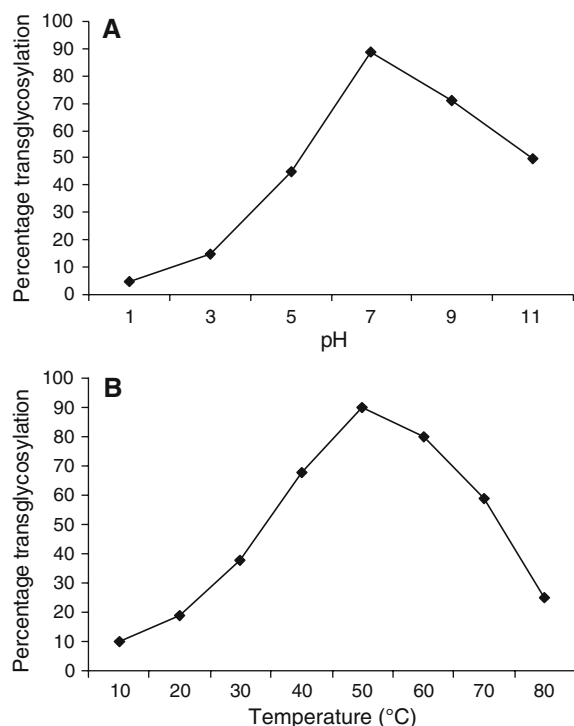
Fig. 5 Effect of pH and temperature on transglycosylation in the MAR system

Table 2 Effect of the molar ratio of stevioside to β-CD in MAR system

Stevioside to β-CD ratio[a]	Transglycosylation (%) ($n = 3$)
4:1	18 ± 0.3
2:1	38 ± 0.7
1:1	58 ± 0.4
2:3	69 ± 0.3
1:2	90 ± 0.6
2:5	65 ± 0.2
1:3	45 ± 0.5

[a] Stevioside 1.24 mmol, β-CD (0.44 mmol−2.64 mmol) at 80 W, 50°C for 1 min in sodium phosphate buffer (5 ml). Fixing the substrate concentration constant, β-CD as substrate was used in various ratios as glucose donor for standardization of optimum transglycosylation. All the reactions were carried out in MAR system under similar conditions

665.02 respectively thus confirming the attachment of one and two glucose moieties in I and II respectively.

Conclusions

Three different techniques of transglycosylation of stevioside molecule were studied and their yields compared. An efficient 1, 4-intermolecular transglycosylation with β-CGTase in presence β-cyclodextrin as glucose donor was accomplished for the first time under microwave conditions. CGtase from alkalophilic *B. firmus* was found an effective biocatalyst in transglycosylation, removing aftertaste bitterness of stevioside and improving its sweetness index. Microwave-assisted reaction (MAR) proved to be a rapid and convenient resulting in optimum yields of two α-glycosylated biotransformed products I and II in 65% and 25% respectively. MAR is also an environment friendly technique and offers fast alternate to traditional and ultrasound methods with enhanced transglycosylation yields. CGTase from *B. firmus* exhibited specific property of higher transglycosylation rate under MAR system. This technique saves considerable time and energy, It operates at low energy cost and has potential for commercial production of modified de-bittered stevioside.

different temperatures (10–80°C), maximum yield of I and II was at 50°C. This shows that at 50°C the enzyme was very stable in free state under microwave conditions favouring substrate towards binding sites. The optimum ratio of stevioside to β-CD for optimum transglycosylation was 1:2 is shown in Table 2.

Reaction product I and II after purification were characterized by detailed NMR, LC-MS/MS studies and their selective alkaline hydrolysis. Integration of one additional glucose moiety in I was confirmed by ¹H, ¹³C, HMBC and HMQC spectral data. In HMBC, H-1′, H-1″, and H-1‴ correlated with δc 84.9, 79.6 and 176.1 respectively. The downfield shift of C-4′ (δ 9.2 ppm) and HMBC correlation of H-1″ and C-4′ clearly indicated the attachment of additional fourth glucose moiety at C-4′ position after transglycosylation. Further, attachment of two additional glucose moieties in II was confirmed at C-4″ and C-4‴ positions respectively on the basis of ¹H, ¹³C, HMBC and HMQC. Alkaline hydrolysis of I and II resulted in cleavage of glucose moieties at C-19 positions. LC-MS spectra after hydrolysis of I indicated a mass $[M + Na]^+$ of 827.17 and that of II $[M + Na]^+$ of

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