

Sucrose-induced biosynthesis of pharmaceutically important secondary cell products in cell suspension cultures of *Stevia rebaudiana* (Bert.)

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Research Article

Keywords: antioxidant, cell culture, polyphenolics, stevia rebaudiana, steviol glycosides, sucrose feeding

Posted Date: September 10th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4877810/v1>

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Additional Declarations: No competing interests reported.

Abstract

Stevia (*Stevia rebaudiana*) is well known for production of pharmaceutically important and nontoxic steviol glycosides (SGs) which can be used as alternative sweeteners to regular sugar. The primary focus of this report was to investigate the sweet diterpenes in cell suspension cultures with different growth responses to sucrose. Initially, different concentrations of carbon source (sucrose; 05–50 g l⁻¹) were tested for the establishment of cell suspension culture and growth kinetics were determined for 30 days period with three days intervals for each treatment. Cultures collected at late-log phase (day-18) were used for determination of steviosides, rebaudioside-A, dulcoside-A, total phenolic content, total flavonoid content and antioxidant activity. The results showed maximum values for fresh biomass (97.71 g l⁻¹-FW), dry biomass (8.57 g l⁻¹-DW), rebaudioside-A content (27.64 mg/g-DW) and dulcoside-A content (6.43 mg/g-DW) in response to 20 g l⁻¹. The cultures grown with 30 g l⁻¹ sucrose exhibited maximum stevioside content (42.23 mg/g-DW) and highest antioxidant activity (83.87%). However, the exposure of suspended cells to sucrose (40 g l⁻¹) improved total phenolic (139.20 mg/g-DW) and total flavonoid (41.46 mg/g-DW) contents. The correlation studies showed strongly positive correlation of dry biomass and phenolics and flavonoids contents respectively. Furthermore, the antioxidant activity was positively correlated with phenolics and flavonoids contents, but not with steviol glycoside content. This work shows the potential for utilizing liquid suspension cultures of *S. rebaudiana* for enhanced production of SGs and antioxidant phenolic metabolites.

Introduction

Stevia rebaudiana, a perennial sweet herb [1], is one of the natural substitutes of regular sugar and free from calories [2]. A special group of compounds (diterpene glycosides) are synthesized in the leaves of *Stevia* which are 300–400 times sweeter than table sugar [3–6]. Moreover, Kim et al. (2011) [7] reported that phenolic phytochemicals and some vitamins as natural antioxidants in *Stevia* play a key role as chemopreventive agents to control cellular oxidative damages. The dried leaves of *Stevia* are rich source several valuable secondary metabolites such as alkaloids, xanthophylls, flavonoids, hydroxycinnamic acids and water-soluble chlorophylls [8]. Nevertheless, various elicitors (biotic and abiotic) as stresses fluctuate or regulate the biosynthetic pathways of these secondary metabolites [9].

The content and composition of SG, however, vary widely between genotypes [10]. To enable scalable production of the desirable SGs from superior genotypes, the use of SG production from engineered yeast has recently been explored [11]. Another more straightforward approach is to produce SGs from *in vitro* clonally propagated plant cultures, such as cell suspension, derived from the superior genotypes.

Different *in vitro* techniques for production of plant material have been extensively applied to produce important plant-produced compounds on commercial levels in most medicinally valuable plants [12]. Cell suspension culture arguably has the highest potential for large scale production [13] as they grow fast and can be utilized for continuous production of the targeted bioactive compounds [14] utilizing well established fermentation technology for scale-up [15]. Additionally, cell cultures can also provide useful

research material for fundamental studies on biosynthetic pathways [16]. Wu et al. (2007) [17] and Sivakumar et al. (2011) [18] reported that the most reliable system for uniform production of berberine, ginsenoside and shikonin in high-valued plants are cell cultures. However, limitations still exist resulting in poor cell growth and low yield of metabolites [19]. Cell growth and metabolites production have been directly linked to medium composition [20] precursor feeding and elicitation [21]. The production of bioactive compounds in cell suspensions can be stimulated by exposure to biotic and abiotic elicitors [22, 23]. Abiotic elicitors include quality and quantity of light, pH levels and sucrose at various concentrations [24].

Sucrose is the key source of energy and carbon for plants both in *in vivo* and *in vitro* conditions [25]. The phloem tissue of higher plants contains sucrose that regulate gene expression and osmotic potential [26–28]. Like other salts, sucrose also acts as osmotic agent which play a key role in the movement of various components across the cellular membrane in suspension culture. Hence sucrose regulates the biosynthesis of secondary metabolites as well as the absorption of various components from the culture [29, 30]. Additionally, sucrose improves plant resistance level [27, 28] by inducing antioxidant secondary metabolites under stress conditions [31, 32]. Cultured plants *in vitro* of *Stevia* responded to different levels of sucrose in the culture medium in terms of altered content of SGs and transcriptional regulation of genes involved in the SG pathway [33]. However, limited information is available on how sucrose interacts with the biosynthetic pathways involved in the SG response [5, 34].

The main aim of the current report was to examine the differential influence of various sucrose concentration on biosynthesis of secondary cell products (different SGs, phenolics, and flavonoids) and to investigate the suspended cells of *Stevia rebaudiana* for antioxidant activities.

Materials and Methods

Establishment of suspension cultures and growth kinetics

For callus induction, leaf explants from 30-day old *in vitro* seed derived plantlets were inoculated onto MS (1962) media supplemented with the combination of benzyl adenine (BA) and 2,4-dichlorophenoxyacetic acid (2, 4-D). The concentrations of 2, 4-D and BA in the culture media ranges from 0.5, 1.0, 1.5, to 2.0 mg l⁻¹ in Erlenmeyer flasks (500 ml). The friable callus obtained in response to the synergistic effect of 0.5 l⁻¹ BA and 2.0 mg l⁻¹ 2, 4-D and further this combination was exploited for the development of cell cultures of *Stevia rebaudiana*.

To optimized the suspended cultures of *Stevia*, 6 weeks old friable and granular callus cultures were transferred to fresh MS liquid media (8 g l⁻¹) containing the combination of BA and 2, 4-D having varying concentrations of 0.5, 1.0, 1.5, and 2.0 mg l⁻¹ respectively. To obtain optimum response, these *Stevia* cultures were exposed to continuous shaking (rotary shaker) at 120 rpm and 25°C in the dark for 2 weeks. The maximum biomass was accumulated in response to 0.5 mg l⁻¹ BA and 1.0 mg l⁻¹ 2, 4-D.

This plant growth regulators combination was used to establish suspension cultures for the sucrose experiments. The PGRs-free MS media was used as control.

To find out the effect of sucrose, cell suspension cultures were established by culturing 0.5 to 0.8 g of friable calli in 50 ml liquid MS media, supplemented with combination of 0.5 mg l^{-1} BA and 1.0 mg l^{-1} 2, 4-D, and with different sucrose treatments ranges from 5 to 50 g/L (increment 5 g/L). The suspended cells were investigated in 100 ml Erlenmeyer flask, with 27 flasks for each treatment. The flasks were cultured for 30 days on an orbital shaker at 120 rpm in the dark. Fresh weight of biomass was measured from each treatment every 3 days and lag, log and stationary phases for the different experimental treatments determined. At the late log phase (18-day old culture), fresh weight (FW) and dry weight (DW) of biomass were determined and oven dried (50°C ; 24 hours) cultures were used for determination of antioxidant phenolic, flavonoids and SGs.

Analytical methods

Extract preparation

For extract preparation, all oven dried samples were ground with pestle and mortar to a fine powder. 20 mg of each finely powdered sample was dissolved in 20 ml ethanol. Mixtures were incubated in at 25°C to extract out maximum metabolites from powdered samples. After one week of incubation, all samples were centrifuged (15 min; 5000 rpm). Supernatants of the centrifuged samples were further analyzed for total antioxidant potential (DRSA), stevioside, rebaudioside-A and dulcoside-A contents, total flavonoid content (TFC) and phenolic content (TPC).

Determination of total phenolic content (TPC)

TPC was investigated in each treatment according the latest protocol of [35]. Briefly, Folin-Ciocalteus (2.25 ml; 2N) was mixed with ethanolic extract of a sample (0.1 ml), followed by addition of 20% Na_2CO_3 . After incubation in the dark for two hours, the absorbance of each reaction mixture was monitored on Shimadzu-1650 (Japan) UV-Visible spectrophotometer at 765 nm wavelength. Gallic acid (GA; Sigma; Germany) was purchased as standard for phenolics determination. Different concentrations ($1.0\text{-}1000 \mu\text{g/ml}$; $R^2 = 0.9878$) of GA was applied for the establishment Calibration curve. After plotting the curve, the TPC concentration was calculated as GA-equivalent-mg/g-dry weight of each sample by exploiting the following formulae:

$$\text{Total phenolics content} = (\text{A-Sample-A-Blank}) / (\text{Conversion Factor} \times \text{Dilution Factor})$$

Where “A” demonstrates the absorbance of both samples at 765 nm wavelength

Total phenolic production (TTP: mg l^{-1}) was calculated by multiplying total phenolic content (TPC: mg g^{-1}) and dry biomass (g l^{-1}).

Determination of total flavonoids content (TFC) in suspended cells

For TFC determination, aluminum chloride calorimetric method was used [35]. Briefly, ethanolic extract of sample (0.25 ml) and AlCl_3 (0.2 ml; 5% w/v) were mixed and the final volume was adjusted to 25 ml by adding ethanol. For blank preparation, acetic acid (a single drop) was mixed with 0.25 ml of each sample extract and 25 ml of final volume was prepared by dilution. The solution was monitored using 415 nm wavelength by UV-Visible-spectrophotometer (Shimadzu-1650-Japan). The standard for flavonoids (Rutin; Sigma-Germany) was used to plot a standard calibration curve (1.0-100 $\mu\text{g/ml}$; $R^2 = 0.9866$) using the same procedure. The total flavonoid content was expressed as RE-equivalent-mg/g-dry weight of each extract.

Total flavonoid production (TFP: mg l^{-1}) was calculated by multiplying total flavonoid content (TFC: mg g^{-1}) and dry biomass (g l^{-1}).

Steviol glycosides (SGs) contents determination

An established protocol of Aman et al. (2013) [36] was used for High-performance liquid chromatography (HPLC) based quantification of stevioside, rebaudioside-A and dulcoside-A in the suspended cells of Stevia. Further, the HPLC equipment (Perkin-Elmer; USA) was adjusted with various parameters to obtain maximum SGs content. The system was attached with ODS- C_{18} column having 150×4.6 mm size and with particle size of 5 μm . The system was equipped with internal solvent vacuum degasser, detector (with variable wavelength), quaternary pump and the maximum injection volume was kept as 10- μl through auto sampler. For mobile phase, here two different solutions were used as solution A containing HPLC grade water (25%) and solution B having acetonitrile (75%) respectively. Moreover, 10 μl of each sample was injected to the HPLC system with constant flow rate (1.0 ml min^{-1}) to quantify different SGs. However, before the injection of each sample, the single standard (Sigma; USA) for all three SGs was used to obtain standard chromatogram. The standard of SGs was initially used for the establishment of standard and to identify the accurate retention time using HPLC system. The retention time of standard was used as reference and subsequently the retention time of each sample of cell suspension culture containing SGs was compared with standard. After quantification of SGs (stevioside, rebaudioside-A and dulcoside-A) the results were shown as mg per gram dry weight. The production of SGs in liquid cultures was calculated by multiplying dry biomass profiles with their corresponding SGs contents and expressed as mg/l of DW.

Antioxidant activity

The antioxidant activity was determined as free radical scavenging assay following the established method of [32]. Here, the extract prepared in ethanol (1.0 ml; Merk) of each sample was carefully poured into sterile cuvette (3 ml) as solution A. The DPPH powdered (0.125 mg) was dissolved in 20 ml (x 4) ethanol in separate sterile test tube as solution B. The absorbance of solution B was tested to obtain the

desired OD for analysis. For the activity the solution A (1.0 ml) and solution B (2.0 ml) was combined in cuvette and incubated for 30 min in dark to prevent oxidation process. After incubation, the solution absorption was monitored on UV-Visible spectrophotometer at 517 nm wavelength using Shimadzu-1650 system (Japan). The final antioxidant activity was calculated in percentage by means of the following equation:

$$\text{DPPH-RSA (\%)} = (1 - \text{AE/AD}) \times 100$$

Where “AE” was used for the sample (solution A + B) absorption at 517 nm and “AD” symbolize the OD of the solution “B”.

Statistical Analysis

Optimization of cell suspension culture growth response to sucrose was carried out in a Completely Randomized Design (CRD) and each treatment was replicated three times. Analysis of variance of replicated values along with standard errors was performed by Statistix software (8.1 versions). Correlation analysis was performed by using the software GraphPad Prism 5. The graphs were designed by using Origin Lab (8.5) software.

Results and Discussion

Biomass accumulation

To optimize fresh biomass accumulation, it is essential to identify the concentration of sucrose for optimal cell division [37]. The uptake and cellular movement of different inorganic ions in the culture media are dependent on the concentration of sucrose [38] and its further helps in cell maturation, differentiation, and organ development [39]. Among different carbohydrate sources, optimum sucrose is one of the best sources of carbon for in vitro cells but any increase/decrease in the sucrose level act as osmotic agent [40, 41]. In one of the reports it has been observed that the maximum concentration of sucrose in the culture media reduced the growth of cells as well as biomass accumulation [42].

The biomass gain of suspended cells of *S. rebaudiana* was found to be highly influenced by sucrose concentrations. The morphological features of 18-day old cultures are shown in Fig. 1. The log phase of growth occurred between 12–18 days for all sucrose concentrations where the highest fresh biomass accumulations occurred at day-18 in cultures supplemented with 10, 15 and 20 g l⁻¹ sucrose (Fig. 2). This is similar to the optimal sucrose concentration identified for other species. The addition of low-level sucrose concentration (15 g l⁻¹) to the culture media is optimum for cellular metabolism of suspended cells of some medicinal species as initial carbon source for provision of energy [43, 44].

In this study, enhanced biomass accumulation was observed in response to lower sucrose concentrations (5–20 g/l). Maximum fresh and dry biomass (97.71 g l⁻¹-FW and 8.57 g l⁻¹-DW) profiles were displayed by culture media supplemented with 20 g/l sucrose and lowest profiles were observed in response to 50 g l⁻¹ sucrose (Fig. 3). The discrepancy between fresh and dry biomass at higher sucrose concentrations may be explained by the osmotic effects from sucrose, as was observed in *Melastoma*

malabathricum suspension culture where higher sucrose levels (60 g l^{-1}) reduced biomass by increasing osmotic potential [45]. Similarly, reduced biomass accumulation was observed in suspensions of *Zingiber zerumbet* cultured with $25\text{--}50 \text{ g l}^{-1}$ sucrose [46]. Cell growth in cell suspensions of strawberry cultured with $> 30 \text{ g l}^{-1}$ increases the medium viscosity which promoted the osmotic potential in the medium which directly affected the uptake of multiple nutrients [47].

Phenolics and flavonoids accumulation

Different levels of sucrose in the culture medium were shown to induce different concentrations of phenolics and flavonoids (TPCs) in the harvested Stevia cell suspension tissue. The TPC in the DW biomass of suspension cultures varied and dependent on sucrose concentration. The lowest sucrose level (05 g l^{-1}) synthesized 51.34 mg/g-DW of TPC, while, at high sucrose concentration (40 g l^{-1}) the TPC biosynthesis was 139.20 mg/g-DW respectively (Fig. 4). The minimum sucrose level (05 g l^{-1}) reduced the biosynthesis of TFCs. However, as the sucrose level in the liquid media was increased (05 to 10 g l^{-1}), a two-fold increment in the biosynthesis of TFC ($34.55 \text{ mg g}^{-1} \text{ DW}$) was recorded. The flavonoids biosynthesis was directly dependent on sucrose concentration in the culture media. In this study the maximum TFC ($41.46 \text{ mg g}^{-1} \text{ DW}$) was observed in response to 40 g l^{-1} sucrose. The correlation studies showed weakly positive correlation of dry biomass with TPC ($r = 0.184$) and TFC ($r = 0.371$). However, a strong positive correlation of dry biomass and total phenolic production ($r = 0.803$) and total flavonoid production ($r = 0.879$) were observed which suggests that the accumulation of phenolics and flavonoids in liquid cultures of Stevia is directly correlated to DW of biomass (Fig. 4).

It has been observed that sucrose as a carbon source play a key role plant defense mechanism during unfavorable conditions and also involve in the gene regulation of polyphenolics (phenolics and flavonoids) biosynthesis [48, 49] [50–53]. [54, 55] reported that the gene regulation of polyphenolics biosynthesis is not restricted to specific tissues but dependent on culture type, plant species and genotype. In our study, sucrose concentration showed a positive correlation with the production of polyphenolic compounds in cell suspension cultures of Stevia. Solfanelli et al. (2006) [56] have reported flavonoid associated gene expression to be sugar-dependent. [54] observed maximum biosynthesis of flavonoids in suspension culture of grapes (*Vitis vinifera*), possibly by sucrose acting as a signal to stimulate flavonoid gene expression [57]. These results are also consistent with several previous studies, demonstrating sucrose-induced production of phenolics and flavonoids in other medicinally important plant cultures [58, 59].

Steviol glycosides accumulation

The sweetness of Stevia plant is primarily attributed to the content of the steviol glycosides (SGs) stevioside, rebaudioside-A and dulcoside-A [34]. The SG content of Stevia suspension cultures can be stimulated by additions to the culture medium of NaCl and Na_2CO_3 [60], proline and PEG [61] and sucrose. In this study, the lowest stevioside concentration ($20.16 \text{ mg g}^{-1} \text{ DW}$) was measured at the lowest concentration of sucrose (05 g l^{-1}) and the highest stevioside content ($42.23 \text{ mg g}^{-1} \text{ DW}$) in

cultures supplemented with 30 g l⁻¹ sucrose (Fig. 5a). An opposite trend was observed for rebaudioside-A where the highest content was observed by the addition of lower sucrose concentration while, the lower content was observed by using higher concentration of sucrose. The addition of 20 g l⁻¹ sucrose to the culture media produced the maximum rebaudioside-A content (27.64 mg/g-DW), in contrast, the addition of 50 g l⁻¹ sucrose level synthesized 7.97 mg/g-DW rebaudioside-A content (Fig. 5b). Likewise rebaudioside-A content, the supplementation of liquid media with 20 g l⁻¹ sucrose synthesized the highest dulcoside-A content in suspended cells which was detected as 6.43 mg/g-DW, in contrast, any fluctuations in sucrose levels (increase/decrease) drastically inhibited the biosynthesis of dulcoside-A content, while, no dulcoside-A content was observed in suspended cells exposed to higher sucrose concentrations (40–50 g l⁻¹; Fig. 5c).

The role of sucrose for production of secondary metabolites has been demonstrated in suspension cultures of several medicinally important plants like *Artemisia absinthium* L. [43] and *Catharanthus roseus* [62] (Knobloch and Berlin 1980). Likewise, sucrose has stimulated biosynthesis of rosmarinic acid in cell suspensions of *Coleus blumei* [63] and of anthocyanin in cell suspensions of *Vitis vinifera* [64] and *Aralia cordata* [65]. Ahmadian et al. (2013) [30] and Fowler (1983) [29] in the culture media fluctuate the biosynthetic pathways of secondary cell products by acting as osmotic agent and also regulate the absorption and uptake of water as well as other important components of the media for cellular growth. Furthermore, sucrose plays a key role in enzymatic defense system of plants by regulating the genes responsible for the biosynthesis of active secondary metabolites (Steviol glycoside; [66]). Nevertheless, the effect of sucrose on the biosynthesis of high-valued secondary cell products in various medicinal plants have been reported in several studies. Misawa 1985 [63] observed that higher sucrose level enhanced the production of rosmarinic acid in cell suspension cultures of medicinally important *Coleus blumei*. In another study optimum sucrose level of 8% (among 4–12%, w/v) promoted the production of alkaloids in suspended cells of *Catharanthus roseus* [62]. Do and Cormier (1990) [64] also observed that sucrose application enhanced the biosynthesis of anthocyanin in suspended cells of *Vitis vinifera* that may possibly due to osmotic potential created by sucrose concentration.

Antioxidant activity and its correlation with secondary metabolites

Several studies have shown a positive correlation between antioxidant activities and contents of polyphenols of Stevia [7, 67–69]. Strong antioxidant activity of leaf extracts of Stevia was linked to inhibition of nitric oxide, superoxide anion scavenging, hydrogen peroxide scavenging activities, hydroxyl radical, DPPH activities [69].

Antioxidant activity, as DPPH radical scavenging activity (DRSA), in Stevia suspension cultures was found to be sucrose concentration dependent. The DRSA increased from 33.28% in 5 g l⁻¹ sucrose to 83.87% in cultures supplemented with 30 g l⁻¹ sucrose (Fig. 6). The activity showed strong positive correlation with TPC ($r = 0.949$) and TFC ($r = 0.989$), indicating their possible involvement in antioxidant activity of Stevia suspension cultures. However, the antioxidant activity was not found strongly

dependent on SGs, as shown by its weak correlation with steviosides ($r = 0.491$), rebaudioside-A ($r = 0.159$) and ducloside-A ($r = 0.109$). This difference may be due to the sucrose induced differential effects on suspension cultures of Stevia. Recently, we have reported antioxidant activity of adventitious roots cultures of Stevia as poorly dependent on phenolic and flavonoid contents [35]. This difference in results may be attributed to the difference in culture type of Stevia.

Conclusions

In this study, sucrose induced osmotic stress conditions were monitored for maximum biomass accumulation and secondary metabolites production in cell suspension culture of *Stevia rebaudiana*. Among different tested concentrations, 20 g l^{-1} was the optimum sucrose level for higher biosynthesis of fresh and dry cellular biomass. Cell suspension cultures accumulated maximum total phenolics and total flavonoids in response to 40 g l^{-1} sucrose. Furthermore, higher antioxidant activity (83.87%) was exhibited by cultures in response to 30 g l^{-1} sucrose. Similarly, lower concentrations of sucrose ($20\text{--}30 \text{ g l}^{-1}$) favored maximum contents of SGs. The antioxidant activity was found to be in linear correlation with phenolics and flavonoids. The present report will help in optimization and scale up studies regarding enhanced production of SGs and phenolics in cell suspension cultures of Stevia in response to differential treatments of sucrose.

Disclosure statement

All the authors declare that they have no potential conflict of interest regarding the current Manuscript.

Availability of Data

Data will be provided upon personal request to the corresponding author.

Declarations

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Author contributions

NA and NA designed the whole experimental work and wrote the initial manuscript, NA: Supervision, HF helped in methodology, MA helped in data acquisition and UE: helped in protocol development, critically reviewed the manuscript and finalize the manuscript for submission and Muhammad Tayyab (MT) help

in experimental designing, FU and GZ helped in data interpretation, conceptualization and fund acquisition

Funding Declaration: The presented study has got no funding from a funding agency.

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Figures

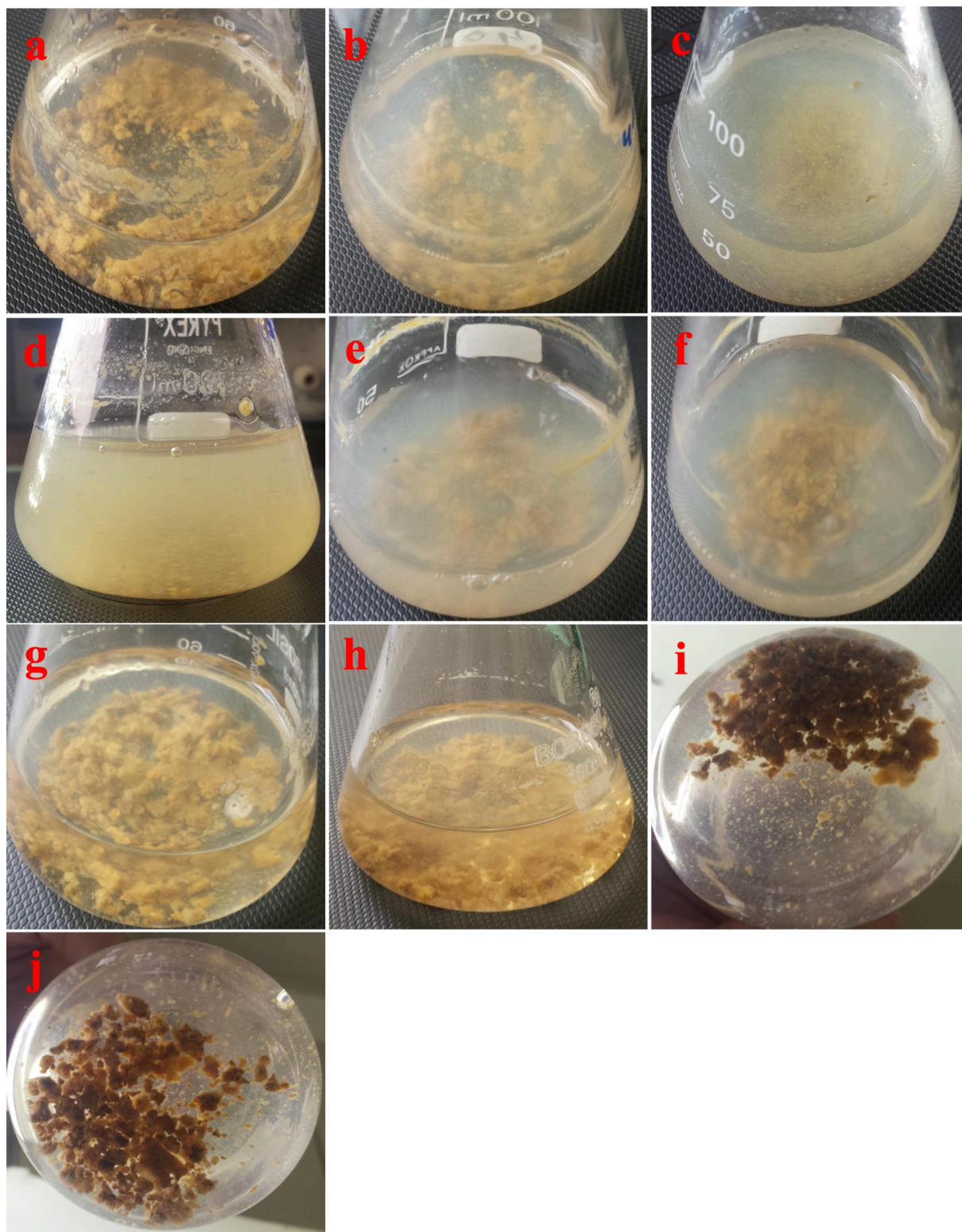


Figure 1

Morphology of late-log phase cell suspension cultures of *Stevia rebaudiana* in response to varying concentrations of sucrose (g l^{-1}) (a) 05, (b) 10, (c) 15, (d) 20, (e) 25, (f) 30 g l^{-1} , (g) 35, (h) 40, (i) 45 and (j) 50 g l^{-1} .

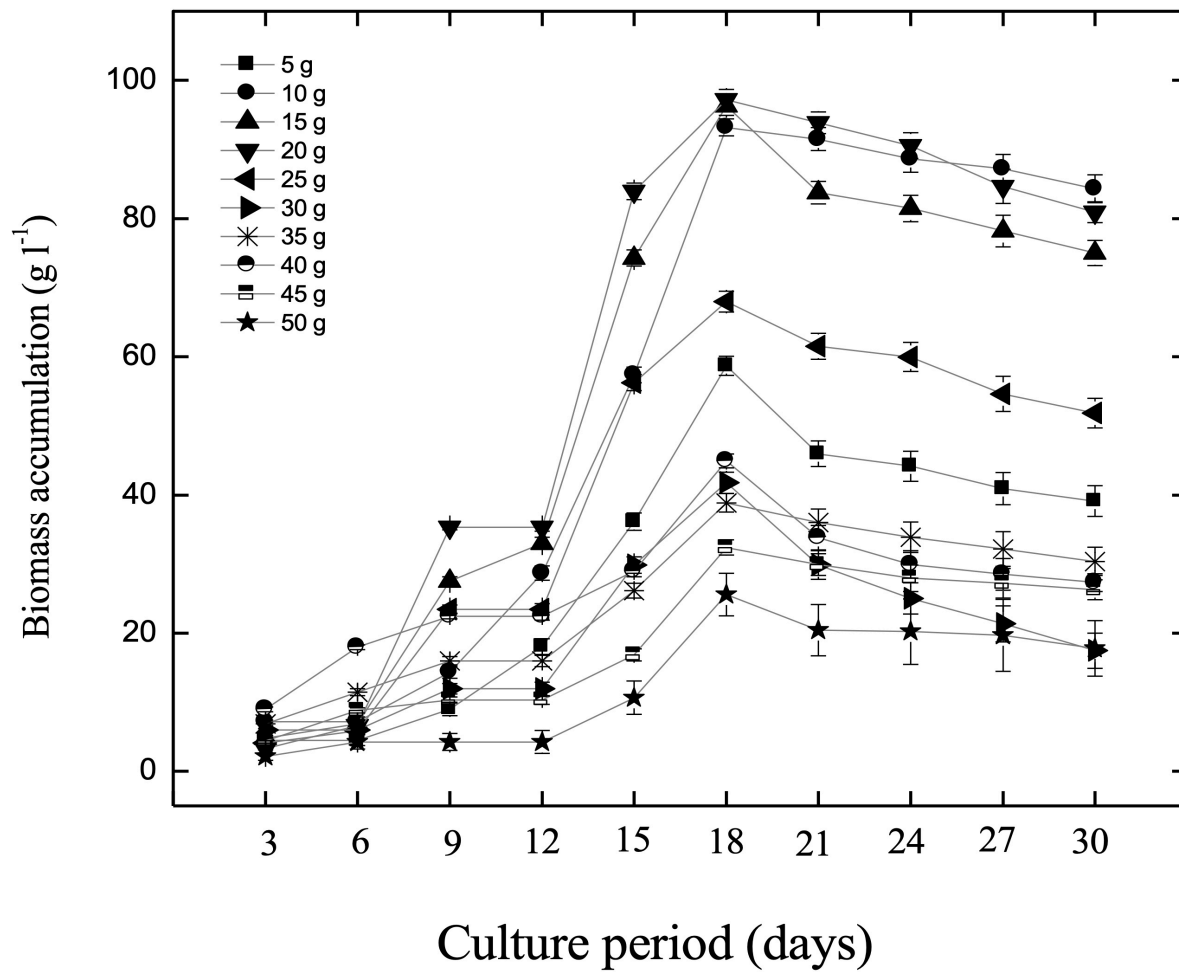


Figure 2

Presentation of growth curve of the suspended cells of *Stevia rebaudiana* (period 30 days; interval 03 days) in response to varying sucrose concentrations (05-50 g l⁻¹).

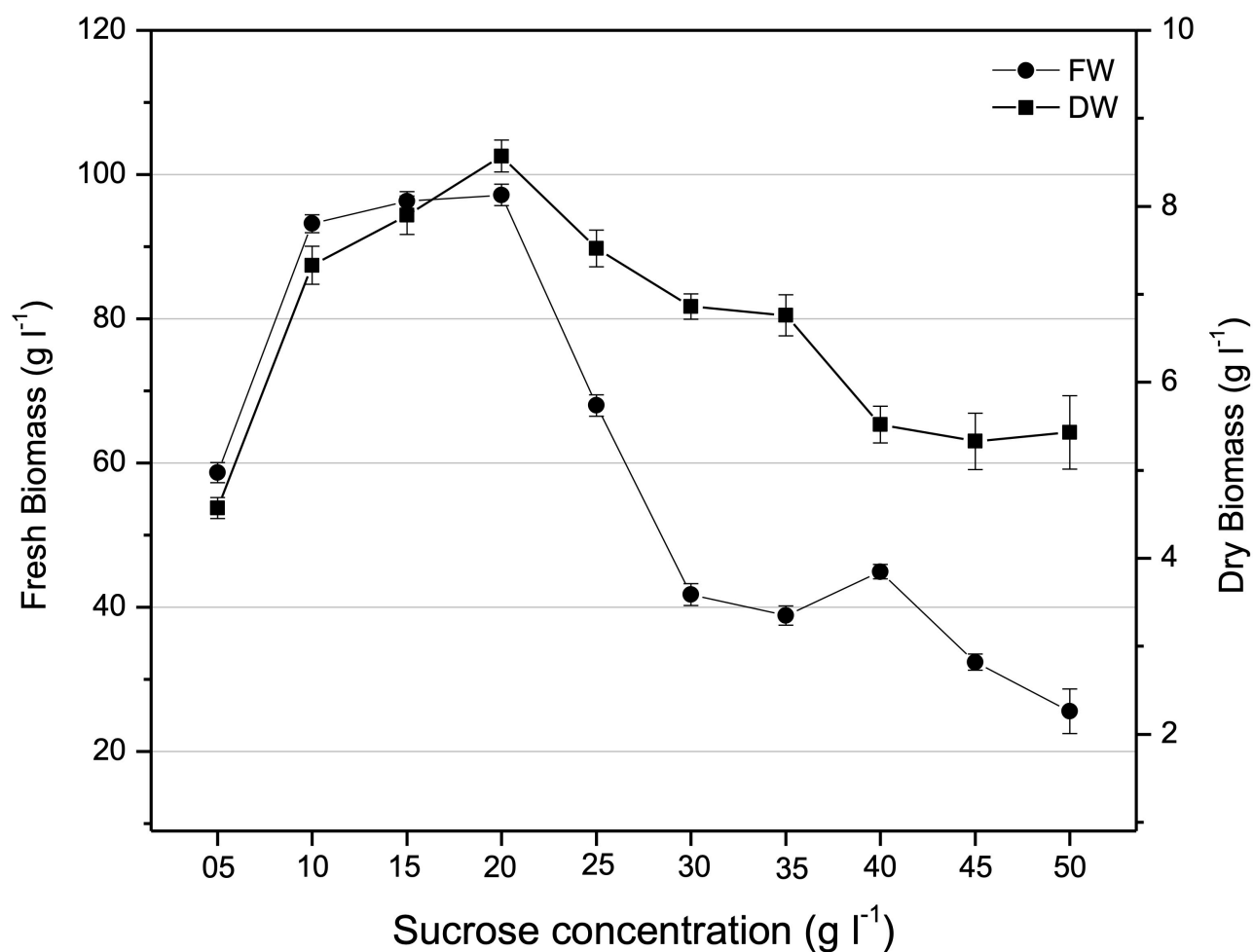


Figure 3

Fresh and dry biomass of 18-day old (late-log phase) cell suspension cultures of *Stevia rebaudiana* cultured with varying sucrose concentrations in cultured media. Mean values ($\pm$ SE) with common letters are non-significant at $P \leq 0.05$.

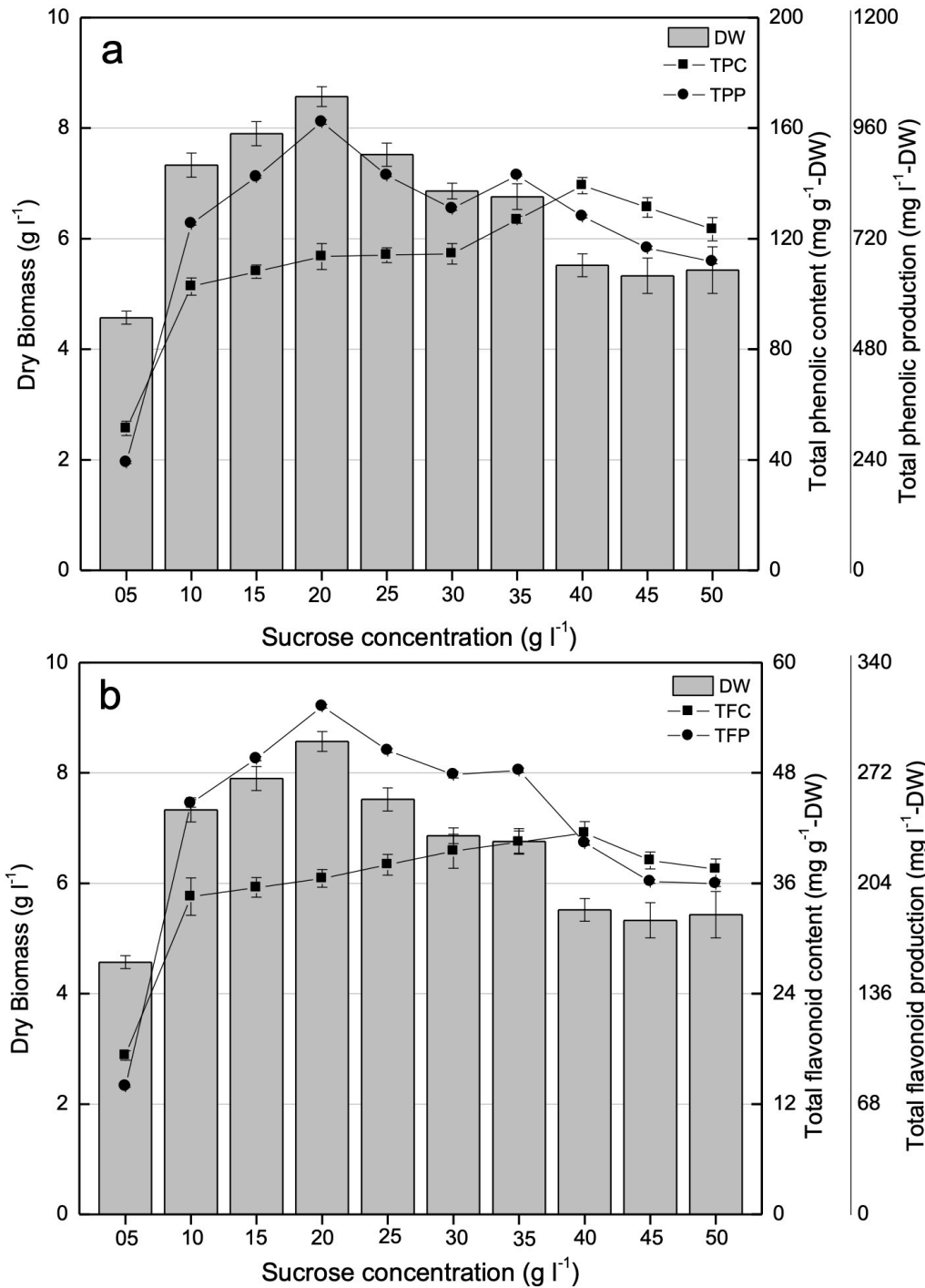


Figure 4

Total phenolic and total flavonoids contents (mg g⁻¹) and production (mg l⁻¹) with respect to dry weight (DW) biomass in cell suspension cultures of *Stevia rebaudiana* cultured with varying concentrations of sucrose ranges from 05 to 50 g l⁻¹. Average values ($\pm$ SE) are significant at $P \leq 0.05$. Pearson correlation (r) profiles: DW and TPC=0.184, DW and TPP=0.803, DW and TFC=0.371, DW and TFP=0.879.

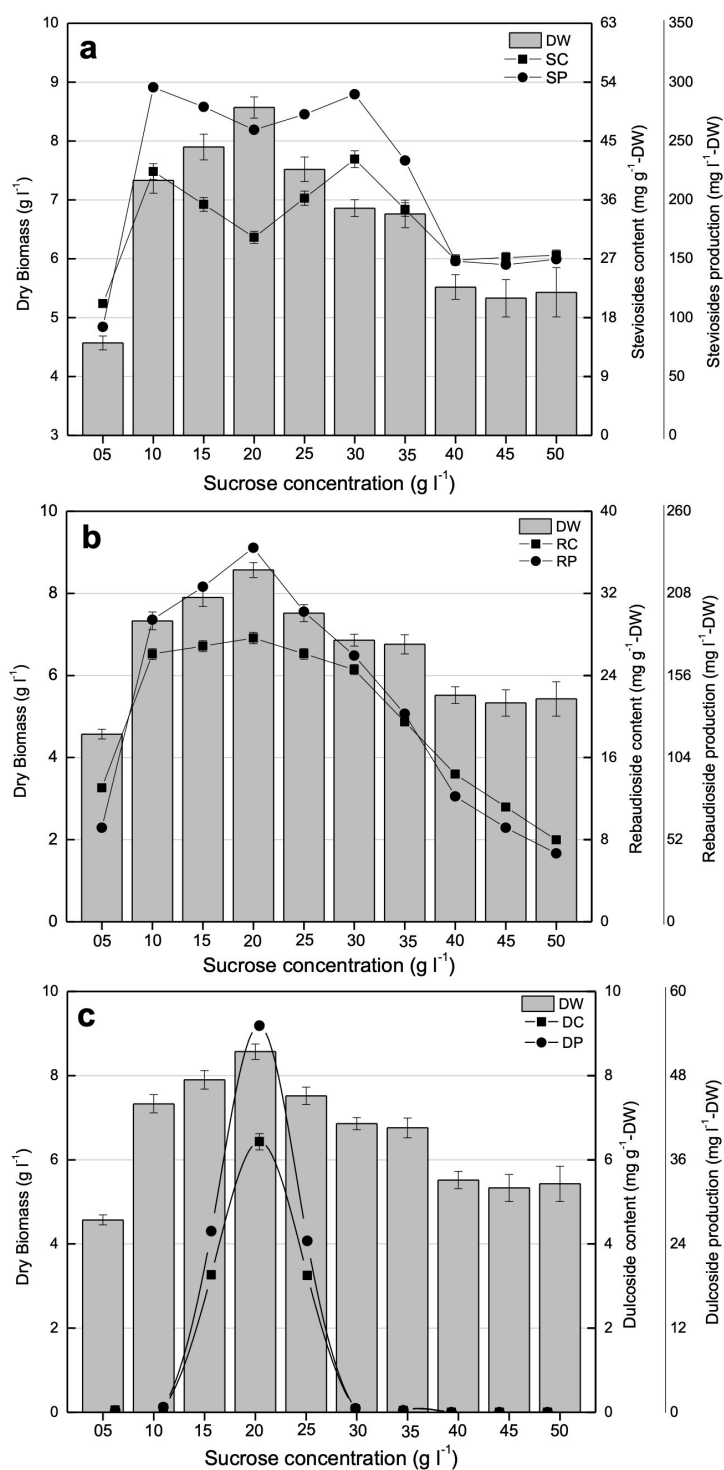


Figure 5

Steviol glycosides content (mg g⁻¹) and production (mg l⁻¹) with respect to biomass in cell suspension cultures of *Stevia rebaudiana* cultured with varying sucrose levels ranges from 05 to 50 g l⁻¹: (a-c; SGs) stevioside, rebaudioside-A and dulcoside-A. Average values ($\pm$ SE) are significant at $P \leq 0.05$. Pearson correlation coefficient (r) profiles: DW and SC= 0.683, DW and SP= 0.901, DW and RC= 0.918, DW and RP= 0.969, DW and DC= 0.768, DW and DP= 0.761.

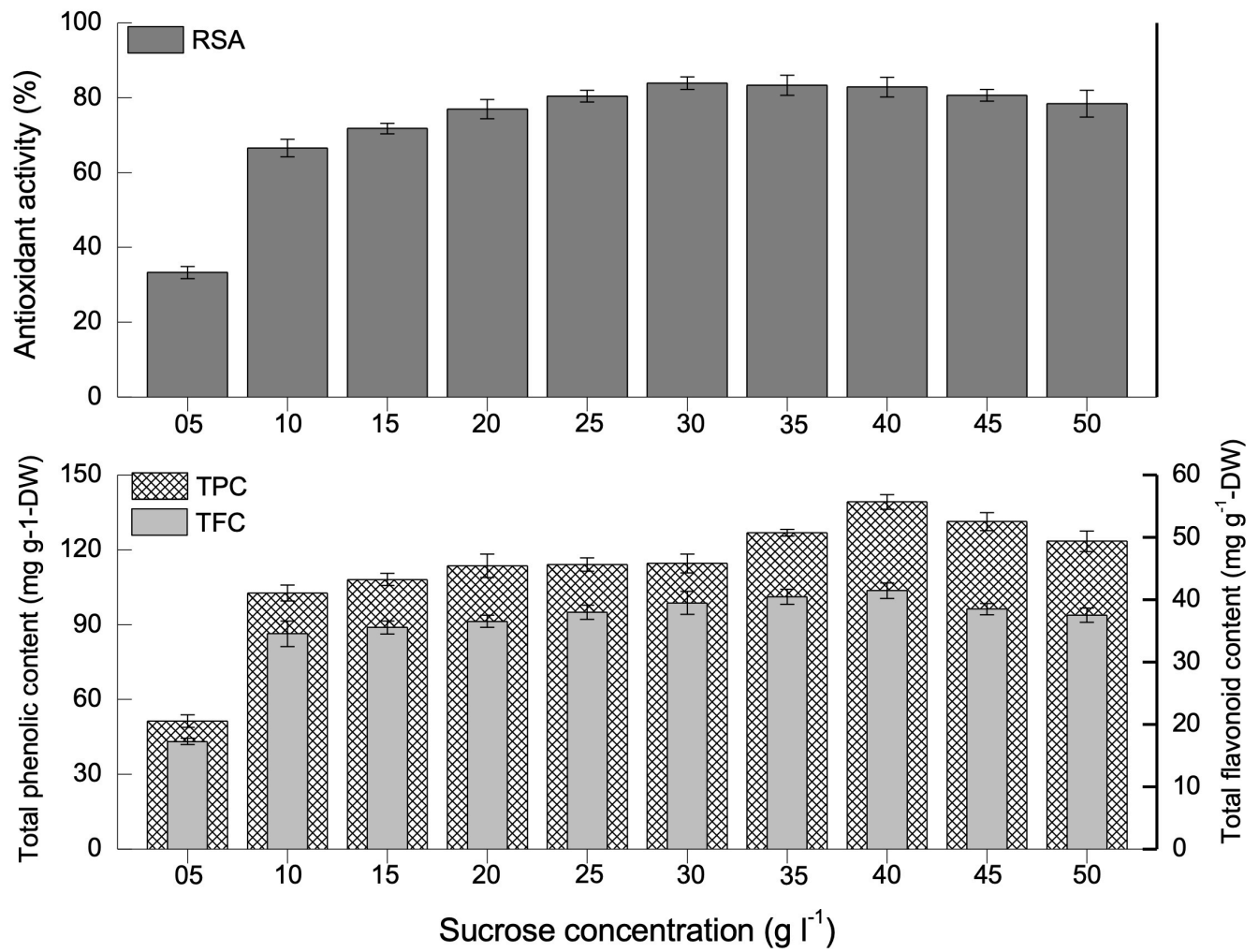


Figure 6

The effect of varying sucrose concentrations ranges from 05 to 50 g l⁻¹ on antioxidant potential in suspended cells of *Stevia rebaudiana* and its correlation with total phenolic content and total flavonoid content. Mean values ($\pm$ SE) are significant at $P \leq 0.05$. Pearson correlation coefficient (r) profiles: RSA and TPC=0.949, RSA and TFC=0.98