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PRODUCTION OF STEVIOSIDE FROM CALLUS AND CELL SUSPENSION CULTURES OF *STEVIA REBAUDIANA* (BERT.)

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ABSTRACT

Stevioside (a non-caloric natural sweetener) is extracted from *Stevia rebaudiana*, which is native plant of South America. In present study, callus cultures of *Stevia rebaudiana* were established on MS medium fortified with different concentrations of BAP, 2,4-D and NAA and their combinations. Stevioside was then extracted from callus cultures by using microwave assisted extraction. Extracts were analyzed for presence of stevioside by using HPLC [C-18 column, flow rate 1ml/min, mobile phase methanol and water (80:20) and detection at spectrophotometer on 201nm]. Callus cultures produced on BAP or NAA supplemented media did not proliferate on subculturing while callus cultures established on media supplemented with 2,4-D produced very hard and brown cultures which were not suitable for cell suspension cultures. Friable green calli were produced on MS medium supplemented with BAP 1 mg/L, NAA 1 mg/L and 2,4-D 2.5 mg/L with maximum quantity of stevioside (33.87 mg per gram of plant material). Cell suspension cultures of *S. rebaudiana* were also established on same media with optimum conditions of pH 5.5, shaking speed of 80 rpm and subculturing interval of 30 days. It was concluded from present study that MS medium fortified with combination of BAP, NAA AND 2,4-D produced green calli of *S. rebaudiana* which can produce stevioside. These green callus cultures can also grow into cell suspension cultures and may be exploited for commercial scale production of stevioside.

Key words: Stevioside, *S. rebaudiana*, Callus, Cell suspension cultures, BAP, NAA, 2,4-D.

INTRODUCTION

Today, world scenario warns about the threat of population explosion where we need more and more land for the production of the staple food for the increasing number of people. There is a regular increasing trend in demand for natural and renewable products. As this agricultural land is scarce, so we need some alternative method to fulfill the requirements of food. One of such alternative is the Cell Suspension Culture (Dicosmo and Misawa, 1995). This method does not depend on the condition or availability of the soil and even on the situation of climate. The controlling factor of this process is just the control of growth and metabolic parameters of the cells which are producing targeted secondary metabolites. This is done in fermenters for commercial scale production of plant secondary metabolites (Fischer *et al*., 1999). It has also been reported in past research work that if growth parameters of cell cultures like light, pH, temperature, aeration and agitation etc are optimized for maximum output, then these cultures would be able to give more products than the native plant (Allan, 1996).

S. rebaudiana, a precious herb and source of a natural sweetener (stevioside), is native to the tropical climates of South America. Its leaves are known to produce an ent-kaurene diterpenoid glycoside (Brandle and Telmer, 2007), which is known to be 110-300 times sweeter than sucrose and have zero caloric value (Cardello *et al.*, 1999; Dacome *et al.*, 2005). Stevioside regulate blood glucose level by increased production and usage of insulin. Stevioside also decreases the blood pressure as it relaxes the capillary walls by decreasing blood cholesterol (Chan *et al.*, 1998). Stevioside is an alternative sweetener to artificial sweeteners like Saccharin, Aspartame, cyclamate, Alitame and Sucralose etc (Anonymous, 2002). Research has proved that these artificial sweeteners, if used continuously, are hazardous to human life. Most of them are carcinogenic but stevioside is non carcinogenic (Toyoda *et al.*, 1997). Furthermore leaf extracts of *S. rebaudiana*/ Stevioside has shown antiviral, antimicrobial, antitumor, antihyperglycemic activities (Jeppesen *et al*., 2000; Jayaraman *et al*., 2008; Kedik *et al*., 2009).

Stevioside is in commercial use in a number of countries as a sweetener and has a huge market value. Stevioside is not easily fermentable and has a longer shelf life. It is also known to be heat stable upto 200˚C and can maintain structure in a wider range of pH. It is not affected by sunlight exposure (Clos *et al*., 2008). It is not even destroyed in taste and color on baking, so it can be used in bakery items without hesitation (Brandle and Rosa, 1992; Mohsen *et al.*, 2002). It has also been reported that it is not needed in purified form for its commercial acceptance (Kaushik *et al*., 2010).

S. rebaudiana has some troubles in propagation through usual propagation methods like seeds or stem cuttings. Naturally, *S.* plants propagate by developing roots on parts of stem which touch soils. Seeds of this plant are small in size, infertile and give very poor germination rates (Dacome *et al.,* 2005). Furthermore, there is another problem with the growth of seedlings which is very slow, ultimately making it impossible to transplant them (Oddone, 1999).

Plant tissue culture and cell suspension cultures are open and gigantic fields now to develop the optimization of a cell suspension cultures' protocol for highest yield of stevioside as for other plant secondary metabolites (Pamela *et al*., 2010). Present study was undertaken on above mentioned facts to exploit the plant through cell suspension cultures for paving the way for industrial level production of the stevioside.

MATERIALS AND METHODS

Plants of *S. rebaudiana* were taken from Botanical Garden of Lahore College for Women University, Lahore, Pakistan. Explants of small size (leaves) were precisely excised and washed with tap water. These explants were sterilized then by using standard protocols commonly used in plant tissue culture experiments.

These explants were then inoculated in solid MS Media supplemented with different concentrations of BAP, NAA and 24D (0.1, 0.3, 0.5, 0.7, 0.9, 1, 1.5, 2 and 3mg/L for each individually). Data were recorded for the days to initiate callus, callus color, texture and proliferation rate after sub culturing. All the concentrations of the phytoregulators, which gave better results for the callus formation and the callus proliferation, were chosen to set up another experiment to show the effect of combinations of these best selected concentrations of the plant growth regulators. Twenty two different combinations were made. Again data were recorded for the days to initiate callus, callus color, texture and proliferation rate after sub culturing. Best media selected was then chosen for the cell suspension culture.

Callus was inoculated in this liquid medium in experiment set up in which different parameters were selected to get the maximum amount of the secondary metabolite (stevioside in the medium) i.e., pH of the medium (4.5, 5, 5.5 and 6), temperature of the culture room (20, 22, 24 and 26˚C), agitation speed (40, 60, 80 and 100rpm) and Interval for subculturing (10, 20, 30 and 40 days).

At the end to compare the quality of callus with reference to the production of stevioside, all the cultures were extracted by microwaves technology using 20% ethanol as a solvent in 200W power level for 120 seconds of irradiation (Javad *et al.,* 2014). Similarly leaves were also taken from the field grown tissue cultured plants and tissue cultured plant from the Lab.

Sample Preparation: 0.1 mg of air dried powder of each sample was mixed with 10ml of the solvent and exposed to the Microwaves.

Quantitative Analysis: Extracts of each sample were dried in open air and then analyzed for the quantity of stevioside on HPLC, using an amino C^{18} column, methanol and water (82:18) as mobile phase and a flow rate of 1ml/min

The experiment was designed in completely randomized design with three replicates. COSTAT V.63 Statistical Software was used for Analysis of variance (ANOVA) and subsequently Duncan's new multiple range test for means comparison (Anonymous, 2009).

RESULTS AND DISCUSSION

Table 1 shows the evaluation of the potential of plant hormones i.e., BAP, NAA and 24D to initiate callus and proliferation response from *S. rebaudiana* using leaf as explants and the production of stevioside. There were two media i.e., MS+BAP 1mg/L and MS+24D 2mg/L which took least time to initiate the callus but the medium mentioned earlier did not show the growth of the callus after subculturing. Results showed that cultures proliferated well after sub-culturing in MS+24D medium. BAP and NAA alone were quite better at some concentrations to begin the callus response but these cultures were not able to proliferate while callus cultures originated in the 24D were compact, so not usable for the cell suspension cultures (Fig 1, 2, S4 and S5).

On the basis of these results, combinations of BAP, NAA, 2,4-D were prepared to get the optimum concentrations for the maximum callus growth of *S. rebaudiana.* Amongst all these combinations, combination 16 (Table 2) was the best combination as it formed the greenish white crystalline callus within an average of 12days and had a high rate of proliferation. It showed the highest amount of stevioside content in it, extracted by MAE and estimated by HPLC (Fig 3, 4, 5 and S3). Combinations 15 and 20 took more time to initiate callus but callus texture and proliferation was suitable to be selected for cell suspension cultures (Fig 6). Present study points out that MS+ BAP 1mg/L+ NAA 1mg/L +24D 2.5mg/L is the finest combination to initiate callus and to proliferate it as well as for the commercial scale production of Stevioside.

It is in accordance with the research work reported by a number of researchers on *S. rebaudiana* and other plants as well. Park and Kim (2003) were of the view that 2,4-D alone in concentration of 1mg/L was more appropriate for the Callogenesis in *S. rebaudiana* while Swanson *et al*., (1992) and Patel and Shah (2009) used BAP and NAA for callusing of *S. rebaudiana*.

Nasircilar *et al*., (2006) and Haensch (2007) also confirmed that 24D produced earlier callus response than other phytoregulators in barley, wheat and datura respectively. They also reported that 24D alone or in combination with other phytoregulators is the main callus stimulating factor in plants.

2,4-D induces the formation of clusters of unorganized cells in the cultures which are known as proembryonic masses (Jong *et al.,* 1993) and it is strongly involved in the process of dedifferentiation (Sane *et al.,* 2012). It is very well reported and understood phenomenon that combination and concentrations of phytoregulators play a very important role in the callus induction and growth (Karimian *et al*., 2014).

If quantity of stevioside from different combinations is compared, one thing becomes clear that the stevioside content is higher in green calli which means these calli have developed their photosynthetic machinery which indicates that *S. rebaudiana* plants require ample amount of sunlight to synthesize stevioside in appreciable quantities. It is in accordance with the results of Bondarev *et al*., (2003) who said that non-differentiated tissue of *S. rebaudiana* were not able to synthesize stevioside so callus and suspension culture have lesser amount of stevioside.

Table 1. Effect of phytoregulators on callus induction and stevioside production from the leaves of honey crop (*S. rebaudiana*)

	Hormone	Treatment (mg/L)	Days to initiation	Callus color	Callus texture	Sub culture	Stevioside mg/g of plant material
1	MS+ BAP	0.1	-	-	-	-	
		0.3	-	-	-	-	
		0.5	31^{d}±0.46	white	Compact	-	13.73
		0.7	29^{fg}±0.50	Pinkish white	Compact	-	14.24
		0.9	25^{i}±0.24	Pinkish white	Compact	-	10.09
		1.0	19^{m}±0.27	Pinkish white	Crystalline	-	08.14
		1.5	-	-	-	-	
		2.0	-	-	-	-	
		3.0	-	-	-	-	
2	MS+ NAA	0.1	-	-	-	-	
		0.3	-	-	-	-	
		0.5	35^{b}±0.26	Yellow white	Compact	-	05.41
		0.7	30^{f}±0.49	Yellow white	Compact	-	09.24
		0.9	29^{fg}±0.39	Brown	Compact	-	09.28
		1.0	31^{d}±0.47	White	Crystalline	-	10.02
		1.5	46^{a}±0.48	White	Crystalline		11.87
		2.0	-	-	-	-	
		3.0	-	-	-	-	
3	MS+24D	0.1	34^{c}±0.49	Brown	Compact	+	11.43
		0.3	31^{d}±0.75	Brown	Compact	+	11.92
		0.5	33^{c}±0.44	Brown	Compact	++	12.56
		0.7	27^{h}±0.45	Brown	Compact	++	12.00
		0.9	21^{k}±0.37	Brown	Compact	++	12.90
		1.0	20^{l}±0.24	Brown	Compact	++	14.22
		1.5	25^{i}±0.20	Brown	Compact	++	14.54
		2.0	19^{m}±0.69	Brown	Compact	++	14.49
		3.0	22^{j}±0.74	Brown	Compact	+++	14.48

Each value is mean of 5 replicates with standard deviation (mean ± S. E). Means within a column not sharing a common superscript differ significantly (P<0.05) according to Duncan's new multiple range test.

Brandle and Telmer (2007) described the bio-synthesis of stevioside in *S. rebaudiana* plant, they described that stevioside synthesis in the plant cell is associated with the development of grana and thyllakoids. It is in accordance with the present study results that green calli produced stevioside in good quantity. Recent reports have also established that all the steps of biosynthesis of the production of steviol from pyruvate are being carried out in chloroplast (Brandle and Telmer, 2007).

Table 3 and 4 indicates the optimization of the medium conditions for the maximum growth of the cell suspension cultures of the *S. rebaudiana.* Optimized conditions were calculated to be pH 5.5, and agitation at

80rpm; and subculturing after 30 days with appreciable amount of stevioside (Fig S1 and S2).

Plant tissue culture is a technique having a number of advantages like greater availability of vigorous, disease free biomass (Pande *et al*., 2000). From an engeeniring perspectives cell suspension cultures have more potential in industrial applications. There are a number of commercial products in market which are being synthesized by cell suspension cultures (Flores and Curtis, 1992; Hussain *et al.,* 2012). As far as production of stevioside from *S. rebaudiana* is concerned, earlier reports showed that there was nill production of stevioside from cell suspension cultures (Nabeta *et al*., 1976). Bondarev *et al*. (2001) accounted that 5-6 times lesser stevioside content of *in vitro* grown *S.rebaudiana* as compared to intact plants. While in little bit earlier reports, Swanson *et al.* (1992) observed that the stevioside content was more in rooted and shooted cultures. Rajasekaran (2007) again reported elevated stevioside content in micro-propagated *S. rebaudiana* plants. Infact culture parameters are very important in determining the viability of cells in culture as well as for obtaining appreciable amounts of plant secondary metabolites.

Agitation speed is very important in plant cell cultures as clumping of cells or cell to cell contact determines the production of metabolite. Furthermore it enhances the mixing and availability of nutrients to all the cells. It also causes the release of exudates in the extracellular environment (Kieran *et al.,* 1995). But over agitation can damage cells due to hydrodynamic stress of cellulosic cell wall, size of cell and number of vacuoles (Drapeau *et al*., 1986). Furthermore for successful cell suspension cultures friable callus is required. Development of active individual cells depends upon plant type, agitation level and phytohormones used (Chattopadhyay *et al.,* 2002).

But this research work has very clear results about the fact that by further optimizing the conditions of Cell suspension cultures on commercial scale for fermenter, an appreciable amount of stevioside can be gained on commercial level through green calli and protocol can be exploited for commercial scale production of stevioside.

Table 2. Effect of combinations of phytoregulators on callus induction and production of stevioside from the leaves of honey crop (*S. rebaudiana*)

Sr. #	Hormone Treatment MS+			Days to initiate the Callus	Callus Color	Texture	Sub culture	Stevioside mg/g of plant material
	BAP (mg/L)	NAA (mg/L)	24D (mg/L)					
1	1.0	0.0	1.5	18^{kwa}±0.67	Brown	Compact	++	13.41
2	1.0	0.0	2.0	18^{k}±0.24	Brown	Compact	++	14.67
3	1.0	0.0	2.5	14^{n}±0.52	Brown	Compact	++	14.94
4	1.0	0.0	3.0	15^{m}±0.63	Brown	Compact	+++	18.30
5	0.0	1.0	1.5	30^{d}±1.41	Brown	Compact	++	15.19
6	0.0	1.0	2.0	28^{e}±0.12	Brown	Compact	++	15.83
7	0.0	1.0	2.5	21^{i}±0.28	Brown	Compact	++	19.34
8	0.0	1.0	3.0	24^{g}±0.38	Brown	Compact	++	20.25
9	1.0	1.5	0.0	40^{c}±0.72	Brown	Compact	--	17.62
10	1.0	2.0	0.0	47^{a}±0.75	Brown	Compact	--	17.65
11	1.0	2.5	0.0	43^{b}±0.27	Brown	Compact	--	19.66
12	1.0	3.0	0.0	41^{c}±0.27	Brown	Compact	--	20.43
13	1.0	1.0	1.0	16^{m}±0.32	Brown	Crystalline	+	19.93
14	1.0	1.0	1.5	23^{h}±0.66	Brown white	Crystalline	++	20.05
15	1.0	1.0	2.0	17^{l}±0.38	White	Crystalline	++++	19.56
16	1.0	1.0	2.5	12^{o}±0.27	Greenish White	Crystalline	++++	33.87
17	1.0	1.0	3.0	18^{k}±0.61	GreenishWhite	Crystalline	+++	25.61
18	1.0	1.5	1.0	20^{j}±0.54	Brown	Crystalline	+++	19.00
19	1.0	1.5	1.5	25^{f}±0.55	Brown	Crystalline	+++	19.89
20	1.0	1.5	2.0	27^{e}±0.53	Brown white	Crystalline	++++	16.43
21	1.0	1.5	2.5	17^{l}±0.32	Brown white	Crystalline	++	17.12
22	1.0	1.5	3.0	21^{i}±0.91	Brown white	Crystalline	++	15.50

Each value is mean of 5 replicates with standard error (mean ± S. E). Means within a column not sharing a common superscript differ significantly ($P<0.05$) according to Duncan's new multiple range test

Table 3. Optimization of the culture conditions for cell suspension cultures of the callus of *S. rebaudiana*

Sr. #	pH	Agitation (rpm)	Percentage yield of biomass
		40	10
		60	11
1	4.5	80	10
		100	9
		120	9
		40	12
		60	15
2	5	80	19
		100	14
		120	12
		40	32
		60	40
3	5.5	80	48
		100	35
		120	24
		40	27
		60	23
4	6	80	23
		100	21
		120	18

Table 4. Optimization of the subculturing interval for cell suspension cultures of the callus of *S. rebaudiana* at pH 5.5 and 80 rpm

Sr. #	Subculturing interval (days)	Percentage yield of biomass
1	20	34
2	25	41
3	30	48
4	35	40
5	40	36
6	45	30
7	50	23
8	55	23
9	60	19

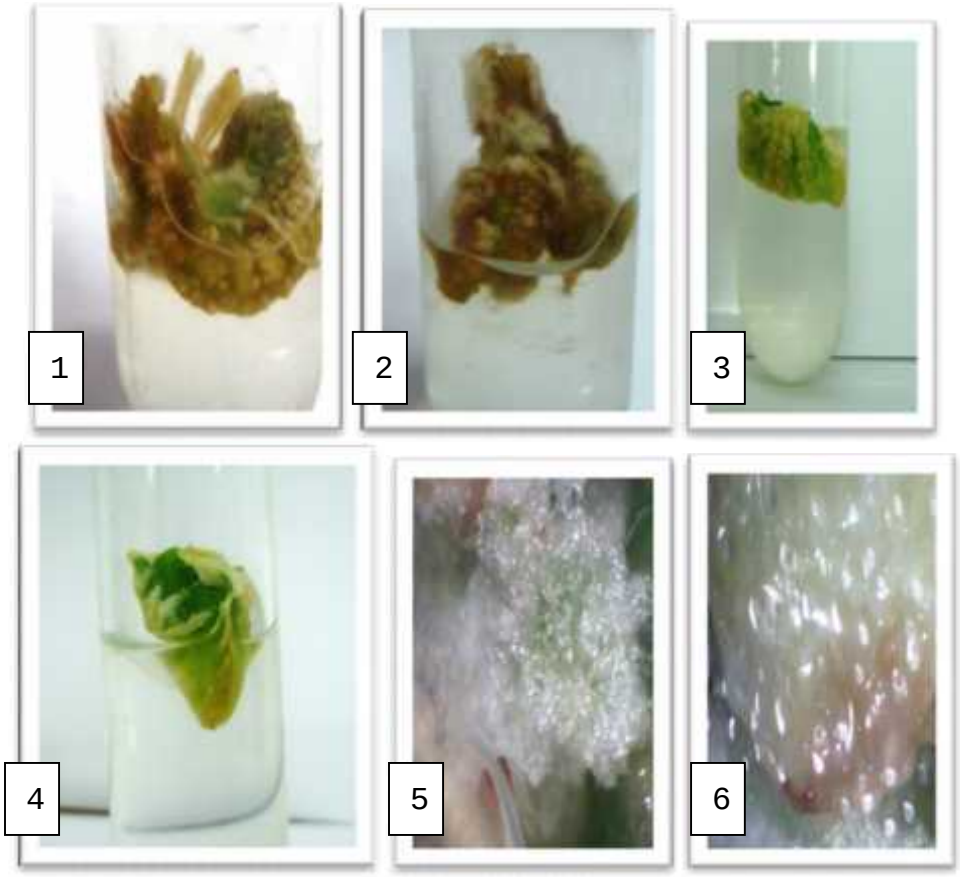

Fig 1 & 2: Callus cultures of *S. rebaudiana* on MS medium supplemented by 2,4-D 3 mg/L
Fig 3 & 4: Callus cultures of *S. rebaudiana* on MS medium supplemented by BAP 1 mg/L+NAA 1 mg/L and 2,4-D 2.5 mg/L
Fig 5: Callus cultures of *S. rebaudiana* on MS medium supplemented by BAP 1 mg/L+ NAA 1 mg/L and 2,4-D 2.5 mg/L by stereo microscope
Fig 6: Callus cultures of *S. rebaudiana* on MS medium supplemented by BAP 1 mg/L+NAA 1 mg/L and 2,4-D 1.5 mg/L

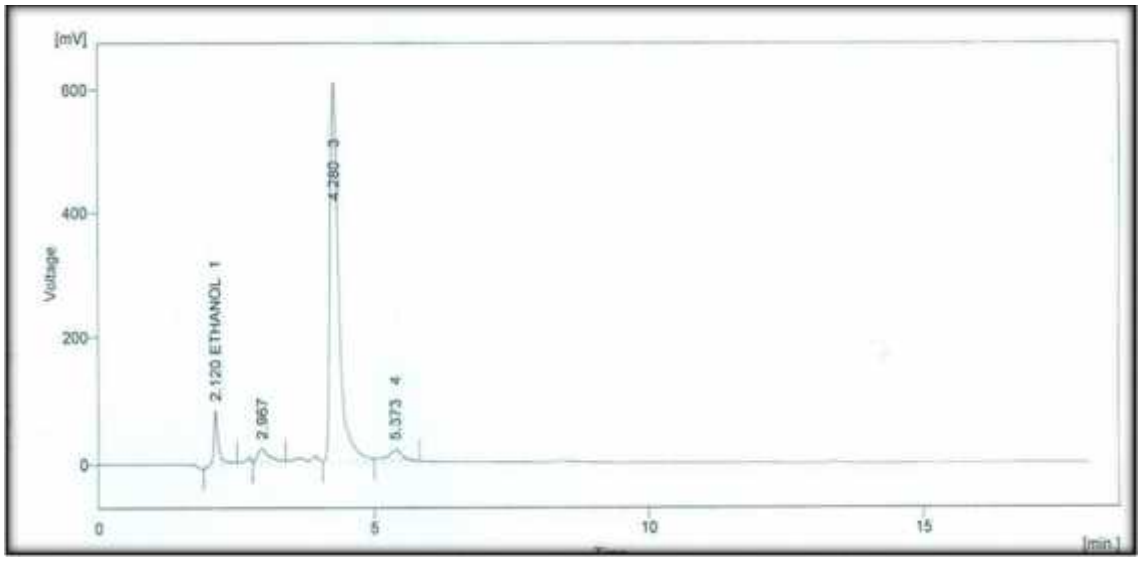

Fig S 1: HPLC chromatogram of standard sample of HPLC grade stevioside

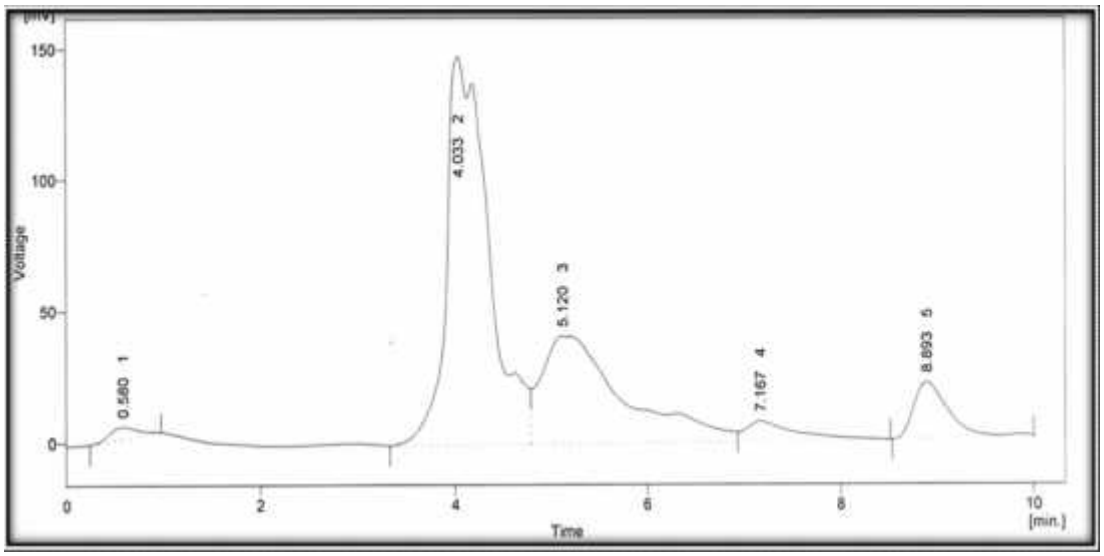

Fig S 2: HPLC chromatogram of cell suspension culture of *S. rebaudiana*

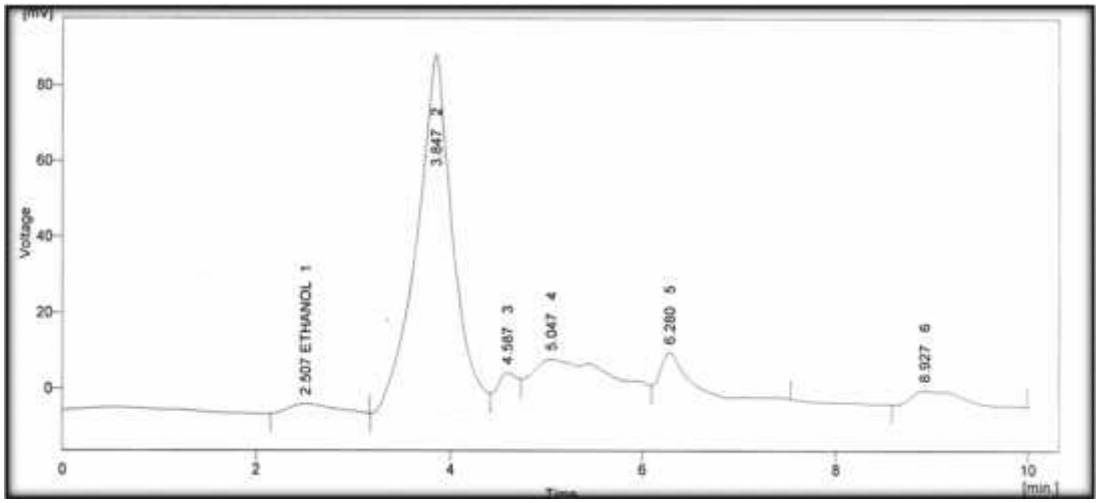

Fig S 3: HPLC chromatogram of callus of *S. rebaudiana* from MS+ BAP I + NAA 1+ 2,4-D 2.5 mg/L

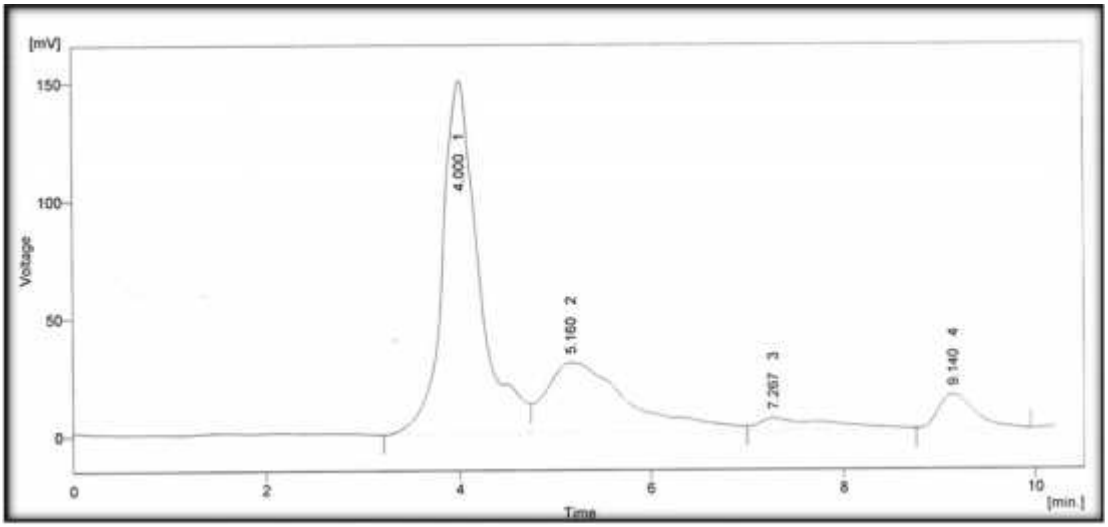

Fig S 4: HPLC chromatogram of callus of *S. rebaudiana* from MS+ 2,4-D 3 mg/L

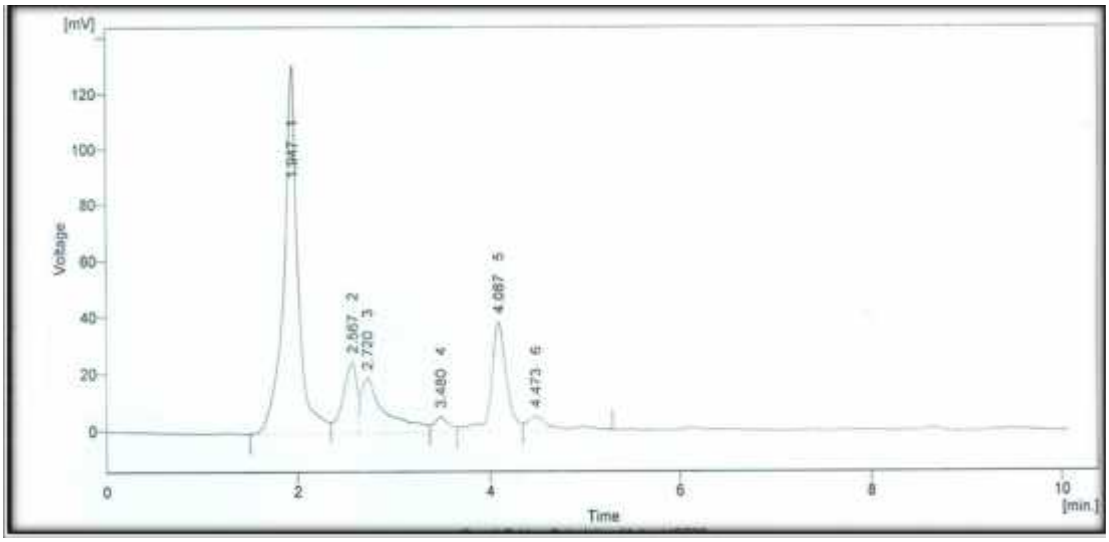

Fig S 5. HPLC chromatogram of callus of *S. rebaudiana* from MS+ BAP 1 mg/L

Conclusion: It is concluded from the present research work that appreciable amounts of green friable callus of *S. rebaudiana* were produced from MS medium supplemented with BAP 1mg/L, NAA 1mg/L and 2,4-D 2.5mg/L. These green calli can be used as the raw material for the commercial production of stevioside by using cell suspension cultures after further optimizing the conditions of cell suspension cultures for fermenters.

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