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Review Article
Critical Review on Steviol Glycosides: Pharmacological, Toxicological and Therapeutic Aspects of High Potency Zero Caloric Sweetener

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Abstract

Stevia rebaudiana Bertoni is a sweet tasting medicinal herb; its leaves are rich source of sweetener "steviosides", which are up to three hundred times sweeter than sucrose, more than half of which is composed by Stevioside and Rebaudioside. Due to its sweet taste it has high commercial value throughout the world as sugar substitute in medicine, foods products and beverages. The increased market share of *Stevia* sweeteners has established a lasting increase in the demand for constant high quality and high purity of *Stevia* products. Clinical examinations performed on Steviol glycosides have shown that it is non toxic and exert hypotensive, cardiotonic, anti-diabetic, anti-carcinogenic, anti-inflammatory, anti-viral and anti-bacterial actions. *Stevia* leaves, steviosides and highly refined extracts of the leaves are now officially used as a low calorie natural sweetener and dietary supplement in many countries. In future, there is possibility that *Stevia* could become a major source of high potency low calorie sweetener for growing demand in natural food market. This manuscript focuses on the phytochemistry, medicinal applications, pharmaco kinetics and safety evaluations of *Stevia* products. Besides this, recent developments in agricultural breeding, biotechnological approaches through cell and tissue culture, improved extraction procedures and biotransformation for taste improvement in *S. rebaudiana* have also been discussed. Future prospects for realization of commercial production of Steviol glycosides are critically evaluated.

Key words: *Stevia rebaudiana*, natural sweetener, medicinal potential, pharmacokinetics, agricultural breeding, biotransformation, extraction

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INTRODUCTION

Stevia rebaudiana Bertoni, a perennial herb belonging to the family Asteraceae, is become a major source of high potency commercial sweetening agent. *Stevia* is known to have originated from Paraguay[1]. The leaves of *Stevia rebaudiana* possesses zero calories sweetening agent, ent kaurene diterpene glycosides commonly known as steviol glycosides which are many fold sweeter than sugar. Studies have shown that these glycosides are non-caloric and can also reduce blood glucose levels, protecting the organism from diabetes and obesity[2]. Moreover, the steviol glycosides has other benefits, such as anti-hyperglycaemic, anti-hypertensive, anti-inflammatory, antitumour, antidiarrheal, diuretic and immunomodulatory effect[3]. The major sweet components found in *S. rebaudiana* are stevioside and rebaudioside A. These representing 90% weight of all sweet glycosides present in the leaves[4]. The other glycosides found in lower concentrations include: steviolbioside, rebaudioside B, rebaudioside D, rebaudioside E and rebaudioside F. The sweet property is remarkably beneficial to diabetic patients and people suffering from obesity. Steviol glycosides are proven to be thermally stable, which makes them more suitable for use in cooked food and drinks. Due to sweet and therapeutic properties of leaf, *S. rebaudiana* has attracted economic and research interests. Toxicological studies based on stevioside have shown that it does not possess tumor inducing or mutagenic effects. *S. rebaudiana* is also known to possess antioxidant activity[5]. Antioxidant delays the oxidation of lipid molecules by inhibiting the initiation or propagation of scavenging oxidative chain reactions. Therefore, attempts are being made to enhance its leaf biomass production through cultural techniques. Above and beyond the realization of various alternative strategies (plant cell and tissue culture), improved extraction procedures and product improvement (taste and flavor improvement) are equally important. The purpose of this review is to corroborate different scientific research conducted on *Stevia* plant to emphasize its remarkable potential as beneficial agricultural crop.

Botanical description: *Stevia* is one of 950 genera of the family Compositae. The genus contains 240 species of plants native to South America, Central America and Mexico, with several species found as far north as Arizona, New Mexico and Texas[6]. *S. rebaudiana* is a small shrubby perennial plant growing up to 65 cm tall[7]. The leaves contain diterpene steviol glycosides, which are estimated to be 300-400 times sweeter than sucrose at a concentration of 4% w/v. *S. rebaudiana* has a very low seed set. The conventional methods of propagation are either by seeds or by cuttings. Since germination rates are poor and seedlings are very slow to establish, it is best grown as an annual or perennial transplanted crop[8]. *Stevia* prefers a well-drained fertile sandy loam or loam soil, high in organic matter with an ample supply of water and partial shade during considerable summer sunshine. Long spring and summer days favor leaf yields and leaf stevioside contents while short days promote blossoming. In horticulture practices *Stevia* is usually propagated by stem cuttings, which root easily. Sweetness in leaves varies with the variety[9].

Phytochemistry and sweetness property: The leaves are rich in tannins and alkaloids, followed by cardiac glycosides, saponins, steroids, triterpenes, reducing compounds and anthraquinones[10]. Stevioside, rebaudioside A, B, C, D, E and F, steviolbioside and dulcoside are diterpene glycosides that are the main sweet constituents of the leaves. Among these, stevioside and rebaudioside A make up more than 50% of the total glycosides[11]. Few new diterpene glycosides have also been isolated and characterized from the leaves of *Stevia rebaudiana*, along with the known steviol glycosides[12-14]. Steviol (*ent*-13-hydroxykaur-16-en-19-oic acid) is the principal aglycone moiety of the glycosides (Fig. 1). The diversity among these glycosides is due to differential glycosylation of steviol by various glycosyltransferases resulting in distinctive physiochemical and organoleptic properties (Table 1). Either a sugar unit or a carboxyl at C19 and either a sugar or a hydroxyl at C-13 is critical for appealing sweetness[15].

Steviol glycoside preparations are white or slightly yellowish, crystalline, odorless powders; these glycosides are freely soluble in water and ethanol and can be easily extracted with an aqueous solvent. The compounds are exceptionally

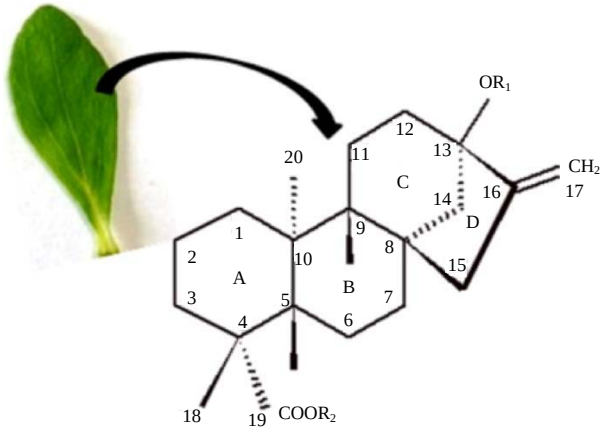

Fig. 1: Steviol: A glycone moiety of sweet diterpene glycosides obtained from the leaves of *Stevia rebaudiana* (R1 and R2 = H)

Table 1: Structure and properties of *Stevia* sweeteners (modified from Prakash *et al.*[14])

Compounds	R1	R2	Melting point (°C)	Potency
Stevioside	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)	196-198	210
Steviolbioside	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)	188-192	90
Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)	242-244	200-300
Rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)	193-195	150
Rebaudioside C	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)	215-217	30
Rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)	283-286	221
Rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)	205-207	174
Rebaudioside F	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)	NA	200
Dulcoside	β-Glc	β-Glc-α-Rha(2→1)	193-195	30

stable at pH values ranging between 2 and 10. In acid solutions, stevioside is highly stable and does not interact with other food components or cause browning[16]. *Stevia* sweeteners can withstand temperatures up to 200°C, unlike sugar which starts to caramelize at about 150-160°C. Even the sweetness of aqueous solutions of stevioside does not change when heated at 95°C for 2 h. The sweetness of stevioside is superior to sugar in mildness and refreshment and the taste lasts for a long period[17].

COMMERCIALIZATION OF STEVIOSIDE AND REBAUDIOSIDE

Stevioside and Rebaudioside A, components of intense sweet taste are the predominant steviol glycosides found in the *Stevia rebaudiana* leaves, serving the purpose of non-caloric sweetener substitute. Besides its sweet property, it also possesses therapeutic benefits.

The commercialization of leaves of *S. rebaudiana* for flavoring and sweetening purposes was first of all introduced in Japan. About 2000 metric tons of dried plant leaves were utilized for the production of stevioside and other sweetener products. Most of the stevioside produced from leaves of *S. rebaudiana* is consumed for sweetening alcoholic beverages[18]. In Brazil, stevioside and the refined extract of plant leaves has been approved for sweetening of medicines, soft drinks, beverages and chewing gum[19,20]. In Paraguay, leaf powder has been used for sweetening of beverages for over a hundred years. In Japan, foods and beverages containing stevioside are easily available. In South Korea, an alcoholic beverage "soju" is formulated using stevioside as a primary constituent and as dietary supplement in United States and some Europian countries.

Despite its widespread use in different parts of the world, there are no reports of its adverse effect in human beings. This situation contrasted with increasing evidence of toxic side effects appearing in the recent literature due to the excessive ingestion of licorice-flavored candy or chewing gum (of which the sweet-tasting triterpenoid glycoside, glycyrrhizin, is a major principle)[21,22].

ABSORPTION, DISTRIBUTION AND METABOLISM OF STEVIOSIDE

In the rat, stevioside is converted to steviol by suspensions of rat intestinal microflora and the conversion gets completed within two days[23]. Stevioside appears to be poorly transported across the cell membrane[24]. No uptake was observed in suspensions of human red blood cells[25]. Previous work has also shown that little or no stevioside is absorbed in the blood of humans instead, all kinds of steviol glycosides get metabolized to steviol and steviol gets absorbed which is also true for Rebaudioside A and Stevioside. This approach has also been validated by JECFA thus, concluding the safe use of stevioside[26-28].

Pharmacological actions

Energy metabolism: Stevioside has been found to interfere with oxidative phosphorylation in isolated mitochondrial cells[29] by disrupting adenine dinucleotide translocation, which is a necessary process in shuttling of high energy phosphate groups generated in mitochondria to their sites of consumption in the cell. Stevioside (5 mM) was found to stop the coupled respiration[27] and it also causes inhibition of mitochondrial ATPase induced by the uncoupling agent,

2, 4-dinitrophenol in rat liver mitochondria. The mitochondrial actions of stevioside have not been observed on intact cells but only reported on isolated organelles. Apart from the effects of stevioside observed on energy metabolism, it has a very little effect on erythrocytes that rely on glycolysis for ATP production[27].

Carbohydrate metabolism: Stevioside is found to reduce the transport rate of glucose into the liver to its half rate. Stevioside also inhibits the release of glucose in hepatic cells. In liver cells undergoing glycogenolysis, the intracellular: extracellular concentration gradient of glucose was found to get enhanced in presence of stevioside.

Effects on blood pressure and renal function: Different research reports based on effects of stevioside have claimed varying effects on kidney function and blood pressure regulation. It lowers mean arterial blood pressure alongwith decreasing renal vascular resistance, produces diuresis and increases fractional excretion of Na^+ and K^+ [30-33]. The lack of effect on glomerular filtration rate implies that stevioside vasodilates both afferent and efferent arterioles[31].

Chromosomal and mutagenic effects: Chromosomal abnormalities have been reported with stevioside at very high concentrations. In a Chinese hamster fibroblast cell line, stevioside did not induce chromosomal aberrations. No chromosomal effects of stevioside were noted in cultured human lymphocytes[34].

Various pharmacological actions of stevioside can be observed on isolated organs and intact cells. It impairs the functioning of kidney disrupting oxidative phosphorylation. Use of stevioside as a food additive has been suggested to control weight gain which might also have toxic effects. However, some reports suggest the use of stevioside in diabetes related obesity. More research is needed on absorption and metabolism of stevioside and also on effect of intestinal microflora converting stevioside to steviol[23].

Rebaudioside A: It's structure is similar to stevioside and differs only in having an extra glucopyranosyl residue attached to the sugar unit at C-13. Rebiana is a purified form of the major glycoside rebaudioside A that meets JECFA specifications along with strict sensory criteria established by the manufacturer. Rebiana can be stably stored as a powder. When stored for 24 months, showed loss of only 1-2% of rebaudioside A[35]. Degradation of rebiana yields steviol glycosides and related steviol compounds which present no safety issues. Rebiana is thermally stable and is also stable in baking products and acidic beverages as compared to other sweeteners. Thus, its stability in acidic beverages makes it more suitable for commercial production of soft drinks.

Efficient hydrolysis of rebaudioside A to steviol has been reported in rat intestinal microflora.

Food and culinary applications: *Stevia* extracts and steviosides are primarily used as a non-caloric sweetener and/or flavor enhancer in a wide range of food products and beverages, like tea, coffee, soft drinks, cordials, weight watcher diets, diabetic diets and fruit juices. As *Stevia* sweeteners are heat stable and do not ferment, they are used in a wide range of products including baked and cooked foods. It has also been used as a source of antioxidants and as an alcoholic beverage enhancer (aging agent and catalyst)[36-38].

Medicinal potential: *Stevia* extracts have medicinal potential as antihyperglycemic, insulinotropic, glucagonostic, hypotensive, anti-cancer, antiviral, antimicrobial, antioxidant, anti-inflammatory, immunostimulatory and chemopreventive agents, as well as for use as a digestive tonic and for dental and skin care[39].

Glucoregulation activity: The traditional use of *Stevia* extract includes treating diabetes as it is found to increase insulin secretion and sensitivity, according to a clinical study[40,41]. The enhancement in sensitivity to insulin influenced by the constituents of *Stevia* leaves might be linked to inhibited hepatic expression of PEP Carboxykinase and gluconeogenesis along with hepatic glycogen synthesis stimulation. Isolated mouse pancreatic islet cells have also shown enhancement in insulin production by the action of Rebaudioside A[42]. Stevioside is also known to promote glucose-activated insulin secretion, without affecting fasting insulinemia[43]. These evidences support healthy glucoregulation activity of stevioside.

Hypotensive activity: Stevioside is able to induce diuresis as well as vasorelaxation and also natriuresis leading to a decline in plasma volume[44-46]. Some studies based on humans have also suggested the role of stevioside affecting cardiovascular system causing hypotension reducing systole duration which could reduce stroke. Long term clinical trials of stevioside on humans have indicated that its continuous consumption can reduce systolic as well as diastolic blood pressure although no significant side effects were observed on lipid or fasting glucose[47].

Antioxidant activity: *In vitro* potential assessment of ethanolic leaf extracts of *S. rebaudiana* have indicated that it possesses antioxidant activity as it inhibits hydroperoxide formation in Sardine oil[48,49]. The antioxidant activity of *Stevia* leaf extract might be due to scavenging mechanism of superoxide and free radical electrons[50].

Antimicrobial activity: *Stevia* has been shown to inhibit the growth and reproduction of bacteria that cause gum disease and tooth decay, making it an excellent addition to toothpaste and mouthwash for dental hygiene[51]. Studies indicate that the major cariogenic organism, *Streptococcus mutans*, experiences growth suppression and secretes less acid when grown on media containing stevioside than when grown on sucrose, glucose or fructose media[52].

Anti-carcinogenic agent: *Stevia* leaf extracts and the presense of polyphenolic constituents have shown inhibitory effect on tumor initiation and promotion. Stevioside, isosteviol, steviol, leaf aglycones and other metabolites are known to inhibit the tumor formation in several ways: by blocking Epstein-Barr virus early antigen, induction[53] and also by reducing production of tumor in two stage mouse skin carcinogenesis model following exposure to 7,12-dimethylbenz[a]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate[54,55].

Anti-inflammatory agent: Anti-inflammatory effects of steviol and stevioside have been observed on epithelial cells of colon[56]. Stevioside has been demonstrated to exhibit inhibitory effects on contraction of smooth muscle of intestine whose inhibition is related to hyper motility-associated diarrhea[57]. According to a study based on cAMP regulated Cl secretion in T84 epithelial cells of colon for observing anti-diarrheal efficacy have indicated inhibition of cAMP activated Cl secretion in T84 cells by steviol along with its analogs[58].

Biosynthesis: A large part of the total metabolism in *S. rebaudiana* is committed to the synthesis of these sweet glycosides making *S. rebaudiana* a good candidate for an EST based gene discovery effort. The biosynthetic pathway of steviol glycoside and its spatial organization has been investigated largely by determining the sub cellular location of several enzymes and elucidation of genes coding for various enzymes in the biosynthetic pathway[59-61]. Recent studies using *in vivo* labeling with [1-13C] glucose and NMR spectroscopy reveal that the precursors of steviol are actually synthesized *via* the plastid localized methyl erythritol 4-phosphate (MEP) pathway and share four steps in common with gibberellic acid (GA_{20}) formation[62]. All the steps up to kaurene occur in plastids, one of the two oxidation steps is located on the surface of the ER and glycosylation takes place in the cytoplasm[63].

Pharmacokinetics: Studies regarding once and repeated use of steviol glycosides have been evaluated in humans[64-66]. The entire steviol glycosides got incompletely absorbed when administered orally but got hydrolyzed to steviol in the colon. Most of the steviol got absorbed and rest was excreted in feces. In liver, glucuronic acid combines with steviol to form steviol glucuronide (Fig. 2). The interspecific difference arises because of the primary excretion of glucuronide which occurs via urine in humans and in rats, via bile[65,67]. No other derivative except for steviol glucuronide has yet been detected in human urine which was exposed to steviol glycosides orally.

Safety evaluations: Steviol and its glycosides have been largely evaluated with a set of *in vitro* and *in vivo* assays, covering aspects of acute toxicity, chronic toxicity, fertility, teratogenicity, nephrotoxicity, hepatotoxicity, mutation, chromosome damage and DNA strand breakage[68]. Additionally, the compounds have been evaluated for nutritional value and allergenicity. Assessments of *Stevia* extract suggested that it contained relatively safe compounds and that oral administration of steviol glycosides had no harmful effects in either animal models or man. *Stevia* extracts and steviosides have no effect on mammalian reproduction or fertility and are safe for use as sweeteners and are acceptable for both diabetic and phenylketonuria patients. Clinical evidence suggested that stevioside can reduce blood sugar levels in type II diabetics and blood sugar in mildly hypertensive patients on long-term treatment with stevioside. Moreover, steviol and steviosides are not carcinogenic nor cancer promoters[69]. Brusick[70] critically reviewed the genotoxicity hazard assessments of various glycosides and reported that steviol glycosides have not been shown to be genotoxic either *in vitro* or *in vivo*. Steviol glycosides have been permitted for use in foods and beverages only in some countries including South Korea, Japan, Argentina, Paraguay and Brazil[65,71]. Other countries permitting the use of steviol glycosides include China, Russia, Indonesia, Mexico (since 2005), Senegal (since 2006), Thailand and Israel. In the USA, steviol glycosides have been allowed as a dietary supplement since 1995. Furthermore, a specific steviol glycoside (rebaudioside A, purity higher than 97%) received no objection letters from the US Food and Drug Administration

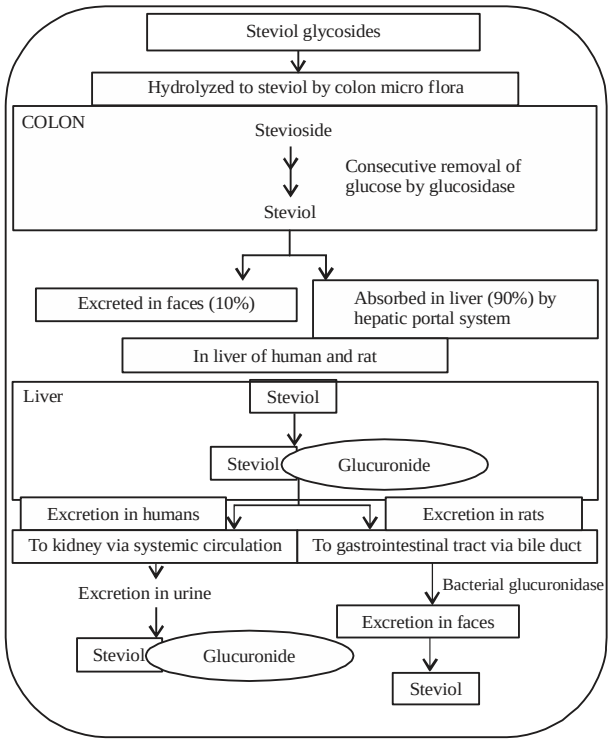

Fig. 2: Schematic depiction of the route from dietary stevioside to steviol glucuronide in humans and rats

(US FDA) (December 2008). JECFA reviewed the safety of steviol glycosides as a sweetener on several occasions (in 2000, 2005, 2006, 2007 and 2009) and established an ADI for steviol glycosides of 4 mg kg^{-1} b.wt., day^{-1} (expressed as steviol equivalents)[72]. In addition to being an approved sweetener in many countries, the World Health Organization has now recognized that stevioside is non-genotoxic and assigned a temporary acceptable daily intake of steviol glycosides of 0-2 mg kg^{-1} b.wt.,[60].

Agriculture breeding: A variety of plant breeding procedures have been used to improve leaf yield and rebaudioside A concentration in plants. In the wild, total glycoside concentration in leaves typically varies from 5-10% on a dry weight basis. Nearly three decades of breeding and selection have increased glycoside concentration to levels as high as 20%[43]. Reports on cultivar descriptions of various countries confirm that sufficient genetic variability exists to make significant genetic gains in leaf yield, rebaudioside A content and the ratio of rebaudioside A to stevioside[73,74]. Therefore, traditional plant breeding approaches such as selection and intercrossing between various desirable genotypes is most suitable method for improving quality traits in crops that are cross-pollinated such as *Stevia*. Various plant varieties with larger amounts of specific glycosides have been developed from selection and intercrossing and patented as RSIT 94-1306[75], RSIT 94-751[76], RSIT 95-166-13[77], T-60[78] and Morita variety (seeds deposited at the International Depository Receipt No. FERM BP-10353)[79]. Due to high degree of natural crossing taking place and also absence of an efficient system of pollination control, part of available heterosis could be captured using composites and synthetics. Synthetics and composites named "AC Black Bird[80] and PTA-444[81] have been developed. Polyploidy produces individuals with better adaptability and increased cell and organ sizes. Experimental breeding of triploid *Stevia* plants resulted in forty-two triploid plants, grouped into eight cultivars with high rebaudioside A or stevioside content[20]. Moreover, the development of molecular marker technology and consequent identification of marker loci linked to important agronomic traits have created exciting new opportunities for plant breeders, overcoming the drawbacks of phenotypic selection which is heavily influenced by environmental conditions and requires selection relatively late in the growing season. Marker-assisted selection provides the potential for improving selection efficiency by allowing for earlier selection and reduced plant population size. A genetic linkage map of *S. rebaudiana* has been developed by analyzing 183 randomly amplified polymorphic DNA (RAPD) markers using segregation data from a pseudo test-cross F1 population[82]. Recent studies on

molecular genetic analysis using the ISSR technique among different *Stevia* accessions revealed a high degree of polymorphism confirming the accuracy of the results. There are about 90 varieties of *S. rebaudiana* developed all around the world. All these have been developed for different climate requirements; as a result, these varieties perform strangely in different climate conditions[67]. Thus, understanding the mechanism and pathway for biosynthesis of steviol glycosides could be helpful in improvement of the glycoside profile by up and down-regulation of genes.

STATUS OF *IN VITRO* PROPAGATION OF *STEVIA*

Stevia has poor seed germination and vegetative propagation through stem cuttings is also limited by the low number of individuals that can be obtained simultaneously from a single plant. Micropropagation of *S. rebaudiana* provides genetically uniform plants in large numbers and with good vigor[83]. Moreover, the tissue cultured plants can be planted throughout the year, except during the peak of summer. The success of *in vitro* plant culture depends mainly on the growth conditions of the source material, medium composition, culture conditions and on the genotype of the donor plant[84,85]. Plants may be regenerated directly or indirectly through callus formation. There are several reports on micropropagation of *Stevia* plantlets with various explants, shoot tip[86], leaf[87,88] and nodal and internodal regions[89,90]. Protocols have been developed to enhance percentage response and number of shoots produced at the induction and proliferation stages[91]. Several experiments have been performed for examining the idiosyncrasy of *in vitro* steviol glycoside production and understanding the way these processes may function in bioreactors for large scale production of this sugar substitute[92]. In addition, plant cell suspension cultures of *S. rebaudiana* have been reported[93,94]. Few attempts have been made to determine the peculiarities of stevioside production in *in vitro* suspension cultures of *Stevia*. Striedner *et al.*[75] reported a maximum concentration of 0.4% of cell dry weight, where the media contained 100 g L^{-1} sucrose after 49 days of incubation. Bondarev *et al.*[95] reported a maximal content of steviosides of 103 g per gram dry weight on the 14th day of cultivation at the end of the exponential phase.

Extraction of *Stevia* sweeteners: All manufacturers use the same basic steps and methodology which involve extraction, purification and separation, to extract steviol glycosides from the leaves, despite the fact that there is some kind of variation in subsequent stages of purification and separation of glycosides[96]. Most commercial products have a total steviol glycoside content of more than 90% with the two main steviol glycosides making up about 80% of the total[71]. Classical techniques used by manufacturers for the extraction of the glycosides include maceration and thermal extraction[97]. In order to increase the yield and quality of the extracted products, several intensification techniques like ultrasonic waves[98], supercritical fluids[99] and microwaves[100] associated with extraction of plant compounds have been developed. Additionally, a multistage membrane process has been developed that is able to concentrate the glycoside sweeteners. The bitter-tasting components were washed out from the sweetener concentrate in the nanofiltration process[55]. A list of patented methods for extraction and purification of steviol glycosides is exemplified in (Table 2). However, membrane based sweeteners can be variable and need to be investigated further.

Biotransformation for taste improvement: Stevioside and rebaudioside A taste somewhat bitter and have an unpleasant aftertaste that limits their application in food and pharmaceutical products. Several attempts have been made to overcome this by modification of stevioside in an intermolecular transglycosylation reaction, catalyzed by various enzymes, during which other carbohydrates are attached at positions C13 and C19[101-103]. Transglycosylating enzymes used for these purposes were pullanase, isomaltase[104], β-galactosidase[105], dextrin dextranase[106] and cyclodextrin glucanotransferases (CGTases) with pullulan, maltose, lactose, partly hydrolyzed starch and cyclodextrins used as donors, respectively. However, the bitterness was removed only in part due to the low yield of derivatives with the required characteristics. CGTases produced by *Bacillus stearothermophilus*, B-5076 and *B. macerans* BIO-4 m serve as effective biocatalyst in the enzymatic transglycosylation of *Stevia* glycosides with the use of starch as a donor[103]. Gtase from alkalophilic *B. firmus* was found as an effective biocatalyst in transglycosylation, removing the aftertaste bitterness of stevioside and improving its sweetness index. A microwave-assisted reaction proved to be a rapid and convenient approach. An efficient 1, 4-intermolecular transglycosylation with β-CGTase in the presence of β-cyclodextrin as glucose donor was accomplished under microwave conditions resulting in optimum yields of two α-glycosylated biotransformed products in 65 and 25% yield, respectively. Moreover, an improvement in taste and quality of glycosides has been

Table 2: Detailed list of patented methods for extraction of *Stevia* sweeteners[77]

Method employed	Part used	Limitations	Publication
Solvents for crystallization	Leaf and stalk	One glycoside is obtained at a time and yields are less. Aqueous methanol is hazardous.	Ajinomoto
Polymeric adsorbent resin and solvents for crystallization	Leaf and stalk	Organic solvents and synthetic adsorbents for purification lead to loss in product and presence of hazardous chemicals in the end product	Maruzen
Polymeric adsorbent, charcoal for depolarization and solvents for crystallization	Leaf	Organic solvents (methanol, acetone) and synthetic adsorbents for purification and traces of hazardous solvent in the end product. At the end, only one glycoside was obtained	Tama Seikagaku
Solvent extraction changed for the feed directly onto anion exchange resin only	Foliage	Solvent extraction and aquafying the extract and treating with only anion exchanger will not give substantial purity to the end product	Maruzen
Water immiscible solvent and methanol is used to purify stevioside	Not given	Organic solvents are used to purify stevioside	Daikin Ind
Synthetic adsorbent and anion exchange resin	Leaves	Adsorbent resins and solvents are used to purify stevioside	Mitsubishi
Different tin salts eg stannous sulfate, stannous chloride, stannic acid etc are used. Precipitates are removed to obtain pure stevioside	Leaf and stalk	Care is not taken about the residual salts and the end product is in an aqueous phase.	Chisso Corp
Different Ca and Fe salts are used. Precipitates are removed toobtaint purified stevioside	Leaf and stalk	Care is not taken about the residual salts and the end product is in an aqueous phase.	Chisso Corp
Solvents like chloroform are used to purify stevioside	Bud and leaf	Toxic chemicals are used	Masuyama
Non polar synthetic adsorbent and solvent used	Leaf	Steviosides have less affinity to these resins and solvents are used to recover them	Sanyo Kokusaku
The extract is optionally treated with ion exchange resins then with adsorbent resins	Leaf	The direct extract loading on adsorbent resin will not give sufficient purity. Moreover, organic solvents are needed for desorption or elution of product. Treatment with ion exchange resins without charging the extract will not give much purity results	Morita Kagaku
The aqueous extract is treated with various salts like $CaCl_2$ and other adsorbents to purify steviosides	Not given	Organic solvent such as *n*-butanol are used	Sanyo Kokusaku
Aqueous extract is treated with acid and then with base and filtrate is treated with polyamide resin and then extracted with n-butanol	Leaf	Acids like H_2SO_4 break glycoside linkages and solvents like *n*-butanol are used to purify stevioside	Yamada Masami
Aqueous extract is treated with $Ca(OH)_2$ and water soluble polymeric flocculent eg. polyacrylamide high polymer to obtain pure steviosides	Leaf	Residual salts may be present in the final fraction	Mitsubishi Acetate
The aqueous extract is treated with $Ca(OH)_2$, $CaCl_2$ while blowing CO_2 gas to remove impurities	Leaf	Calcium chloride is a toxic chemical and residual salts may be present in the final fraction	Mitsubishi Acetate
The leaves were extracted with $Ca(OH)_2$ and water and the extract treated with Amberlite XAD7 and then with ion exchange resins to obtain pure steviosides	Leaf	Amberlite XAD7 treatment is a time consuming step as the steviosides are recovered into organic solvent and solvent is removed and redissolved in water to react with ion exchange resins. Also, Without XAD better results can be achieved	Pacific Chem Ind
Aqueous extract treated with $CaCl_2$ and then with Amberlite XAD-7 and eluted steviosides with methanol and purified by column chromatography and crystallization from methanol	Leaf	Toxic $CaCl_2$ and methanol are used along with column chromatography, which is not a commercially viable option	Pairfir Chemical
Aqueous extract is treated with various membranes like microfilter to obtain pure compounds	Plant	Capital investment on these membrane systems is very high	Canada Nat Res

reported to be increased by transglycosylation using other enzyme systems. These include trans-a-glucosylation of stevioside with maltose and biozyme L (crude α-amylase preparation produced by *Aspergillus* spp.)[107]. *Trans*-3-2, 6-fructofurarylation of stevioside with sucrose and p-fructofuranosidase from *Arthrobactor* sp. K-1 afforded a *trans*-2, 6-fructofuranosylated 19-*O*-glucosyl moiety in high yield from stevioside[102]. Treatment of stevioside with sucrose and glucosyltransferase from *Streptococcus mutans* afforded a better yield than that by the Biozyme L-maltose system[108]. At the same time, Dynamics of stevioside production has been investigated with culture growth in liquid suspensions[55].

SIGNIFICANCE STATEMENT

Despite the recent research advancements and promising results in exploring the genes and enzymes of the biosynthetic pathway, cost effective extraction procedures, improved micropropagation methods and clinical evaluations require serious efforts for improved production of sweet steviol glycosides through modern technology. Biotechnological production in plant cell cultures could be an alternative method which has also been reported in a medicinal herb *Anethum graveolens*[109]. Improvement of process and environmental conditions/or metabolic engineering is key pathways which significantly enhance selectivity and yield of metabolite. However, plant reaction networks are significantly more complex than microbial systems. Pathway redundancy and multiple intracellular compartments complicate flux analysis efforts and manipulation of a single enzyme can lead to unpredictable results. Besides improved production, major concerns regarding commercialization of these glycosides include the issue pertaining to their safety for human use. EUSTAS' specification is very broad; hence additional efforts are necessary to resolve the issues regarding the permitted and safe use of *Stevia* sweeteners.

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