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Review

Critical Review on Key Approaches to Enhance Synthesis and Production of Steviol Glycosides: A Blueprint for Zero-Calorie Sweetener

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Abstract: Steviol glycosides (SvGls) are plant secondary metabolites belonging to the class diterpenes. SvGls naturally derived from non-calorie sugar have therapeutic properties for diabetics, carcinogens, and anti-microbials. Over the past few years, SvGls have been extensively researched because of their extraordinary sweetness without side effects. SvGls are classified into several types based on the number of sugar bases attached to the steviol. Due to the difference in glycosylation, different SvGls have different sensory properties. One of the main obstacles is the mass production of SvGls. Novel physical and chemical treatments and advanced biotechnological approaches are introduced to increase the production of SvGls. Extraction of high-purity SvGls from plants is difficult, so researchers have manipulated the metabolic pathways of engineered microorganisms for bulk production of SvGls. There are many aspects related to the biochemistry and metabolism of SvGls, but their functional relationship with the *S. rebaudiana* is not well understood. Thus there is a need for in-depth research in this area. This review provides the readers with an overview of the research gaps and possible promising methodologies that can be utilized in the future. To trigger more research, this review encompasses the recent developments in SvGls production and marketing.

Keywords: steviol glycosides; biosynthetic pathways; biotechnology; metabolic engineering; stevia commercialization

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1. Introduction

Stevia rebaudiana is a perennial bushy shrub that belongs to the Asteraceae family. Over 200 years ago, the indigenous people of South America used its leaves to sweeten beverages and chewed them for their sweet taste. The stevia plant was first officially recorded in 1899 as *Eupatorium rebaudianum* by the scientist Moises Santiago de Bertoni in the Amambay region, in the northeast of Paraguay (South America) [1]. Later, in 1905 it was renamed *Stevia rebaudiana* after the chemist Ovidio Rebaudi [2]. It is also known as the "sweet herb of Paraguay", honey leaf, candy leaf, and sweet herb [3]. With its increasing popularity, in 1970, for the first-time stevia plant extract was commercially adopted as a natural sweetener by Japan. Currently, stevia is commercially available in China, Brazil, Kenya, the United Kingdom, and several regions in Asia (India, Indonesia Korea, Malaysia, Philippines, and Thailand).

The stevia plant contains secondary metabolite steviol glycosides (SvGls), which are mostly synthesized in leaves and in small amounts in the stem [4]. Steviol glycosides are chemical compounds derived from plant parts and are known for their unique property of sweetness. SvGls have become an important third-generation natural zero-calorie healthy sugar source with additional benefits in controlling several chronic diseases (obesity, diabetes, cardiac blockage, and hypertension) [1]. SGs are not absorbed and metabolized in the human body, making them a low-calorie sweetener [5]. Of more than 230 stevia species known, *Stevia rebaudiana* (Bertoni) is the most popular because of its sweetness [6].

Apart from *S. rebaudiana*, SvGls have been found in three other species: *Stevia phlebophylla*, *Rubus suavissimus*, and *Angelica keiskei*. However, these plant species are of less economic importance as they have a low production rate of SvGls compared to *S. rebaudiana* [7].

SvGls show wide diversity, and there are approximately forty SvGls known so far. SvGls along with some other secondary metabolites, for example, coumarins, flavonoids (19.93 mg/g), phenols (24.01 mg/g), and tannins (56.7 mg/g), and some essential oils have given *Stevia rebaudiana* importance in various medicines and food additives [8]. Twelve SvGls are more popular, based on the number of sugar bases attached to the aglycone core [9]. Generally, the SvGls with more sugar bases (Reb M, Reb D, Reb A) are found to be sweeter than those with fewer sugar bases (stevioside) [10]. Stevioside is the most abundant SvGls found in stevia leaves (5–10%) followed by Reb A (2–4%) and Reb M (<0.1%).

The safety approval of the use of stevia extract from the US Food and Drug Administration (USFDA) in 2008 and European Food Safety Agency (EFSA) in 2011, led to an accelerated interest in stevia-derived compounds. Stevioside, Reb A, and high-purity Reb M ($\geq$95%) derived from stevia are recognized as safe for human consumption (GRAS) [11]. High-grade stevioside and rebaudiosides are now used in many parts of Asia, America, and Europe.

Despite such high global market expansion, the production of high-quality stevia is problematic and insufficient to meet the current demand [12]. Since the very beginning, the aim of cultivators and breeders was the genetic improvement and development of varieties with higher life and yield of SvGls and increased abiotic and biotic stress resistance compared to other commonly found stevia cultivars. However, research is ongoing to find novel stevia-derived compounds and also to identify the key genes regulating the synthesis of desired SvGls with a sweeter taste preferable for food and pharmaceutical industries.

The main aim of this review is to encapsulate all the recent conventional and biotechnological advances made so far to enhance the SvGls production. Earlier studies have described the biosynthesis of SvGls and methods used to stimulate the production of SvGls in *Stevia rebaudiana* [7,13]. However, they fail to cover the current detailed information on the newly identified regulatory enzymes involved in the biosynthesis of SvGls. This review covers a comprehensive explanation, illustrative and tabularized presentation of all the key genes identified and characterized to date, and transcriptome-based analysis used to regulate these gene expressions in SvGls synthesis. Additionally, novel approaches such as agriculture breeding and recent advances in metabolic engineering to enhance sweeter SvGls are discussed in detail in this study. It is very important to know the best methods that can be used to target the key genes involved in the stimulation and biosynthesis of SvGls. Thus, it is crucial to conduct an in-depth and extensive evaluation of the literature available and provide the readers with advice regarding the most current and significant studies in SvGls synthesis. Furthermore, the current status of the marketing and commercialization of stevia will make the readers aware of the present market and probable future expansion of *Stevia rebaudiana* production. The organized information that is being assembled in this review will aid in developing and establishing a more specific and systematic enhancement of the production of desired SvGls.

2. *Stevia rebaudiana* Morphology and Biology

Stevia is an herbaceous plant majorly grown in the perineal and sub-tropical regions. It grows to about 65 cm, and some species may grow up to 120 cm in height. Stevia requires very small amount of nutrients. Generally, 100–120 kg nitrogen and 50–60 kg potassium per hectare is the optimum requirement for the stevia growth. The seed germination is temperature conditional and requires 24 °C for optimal seed germination. Seeds have small endosperm and are dispersed by wind. The fertile seeds of stevia are usually dark colored, while the infertile are mostly pale [8]. Stevia has an extensive root system that entails thicker and deeper roots and fine roots around the soil surface. Its root is the only region where there is no accumulation of stevioside. Stevia plants with a high leaf-to-stem

ratio is desirable, as their leaves contain the maximum concentration of steviol glycosides. It has an alternate leaf arrangement with a small, lanceolate, oblong leaf shape. The shoot is subligneous and pubescent. It is a short-day plant that flowers for 3 months. The tiny white flowers are perfectly gathered in 2–6 florets in corymbs arranged in loose panicles. Seeds of stevia are inside slender achenes about 3 mm in length. The reproduction takes place by the seeds, although the seed viability is very poor and exhibits variability [14]. It is a self-incompatible plant, pollinated by insects, i.e., highly cross-pollinated. The stevia plant is highly sensitive to daylight and requires up to 12–13 h of light for its growth. Day length influences the vegetative growth, leaf biomass, and hence the accumulation of steviol. In mid-longitudinal regions, the short duration of sunlight results in early flowering, decreased vegetative growth, limited seed set, and reduced fruit ripening [15].

3. Steviol Glycosides Chemical Diversity and Biosynthesis

Stevia rebaudiana is also commonly called the "producer of diterpenoid steviol glycosides". Steviol glycosides belong to the diterpenoid group, specifically tetracyclic diterpenoids of plant secondary metabolites. SvGls are non-toxic, non-mutagenic, and highly sweet diterpenoids with commercially important uses in the pharmaceutical and food and beverage industry. By hydrolytic cleavage, these glycosides can be transformed into sugar and a non-sugar component. These glycosides comprise a carbohydrate sugar molecule (glycone) attached to a non-sugar component (aglycone). Glucosides, pentosides, fructosides, and a few other sugar compounds are present as the sweet glycosides of stevia [16]. Different glycone lengths are attached to the core of steviol (ent-13-hydroxyur-16-ent-19-oic acid) at the R1 position at C19 and the R2 position at C13. This is the major reason for the diversity of SvGls. The eleven most common diterpenoids that have been identified in the leaf tissues of stevia include steviobioside, stevioside, rebaudioside A (Reb A), rebaudioside B (Reb B), rebaudioside C (Reb C), rebaudioside D (Reb D), rebaudioside E (Reb E), rebaudioside F (Reb F), rebaudioside I (Reb I), rebaudioside M (Reb M), rubusoside and dulcoside [17,18]. Stevioside was the first steviol to be extracted from the stevia leaves. The steviol glycosides extracted from the stevia plant consist of 80% stevioside, 8% Reb A, and 0.6% Reb C, depicting that stevioside is present in quantities two–three-fold that of rebaudioside. These SvGls vary in the degree of sweetness, which depends on the type and number of sugar moiety attached. The concentration of SvGls varies from one genotype to another and is influenced by the growth conditions. Stevioside are ~200–300 times sweeter than sucrose. In the stevia leaves, the ratio of stevioside and rebaudioside governs the sweetness quality of the stevia genotype. The leaves with more rebaudioside than stevioside give a more desirable taste profile, since stevioside is associated with a slight bitterness, astringency, and menthol taste [19]. Reb-M's significantly similar taste to sugar has increased its popularity in the food industry. It is very beneficial for making sweetness-based products such as candies, where the sweet taste is desirable. Reb M elicits a significantly less bitter taste than Reb A; however, the extremely low extraction rate ($\leq$1%) of Reb M makes it economically unfeasible for its mass production from *Stevia rebaudiana* leaves [10]. Thus, increasing the production and extraction rate of Reb M through genetic engineering is very important for its future applications.

The biosynthesis pathway of steviol glycosides is of immense interest to many researchers, since it is a very complex pathway and shares a common precursor compound ent-kaurenoic acid which is also involved in the gibberellic acid pathway. The SvGls synthesis pathway has previously been described by several researchers; however, it still requires detailed research to better understand the regulation of this multi-step complex process.

The whole process of SvGls is divided into three stages. The initial stage is the methylerythritol-4-phosphate (MEP) pathway, which takes place in the plastid. The condensation reaction between pyruvate and glyceraldehyde 3-phosphate (G3P) is catalyzed by deoxy-D-xylulose 5-phosphate synthase (DXS) to give the product deoxyxylulose-5-phosphate (DXP). The DXS is the first-rate limiting enzyme in the MEP pathway. Further, DXP is converted to MEP by the DXR enzyme [20]. This is the second rate-limiting step

in the MEP pathway and also the regulatory step for isoprene synthesis. Following this, the subsequent generation of the five-carbon building blocks dimethylallyl diphosphate (DMAP) and isopentyl diphosphate (IPP) takes place. The second stage of SvGls synthesis starts with the condensation of DMAP and IPP catalyzed by geranylgeranyl diphosphate synthase (GGDPS) to produce geranylgeranyl diphosphate (GGPP). GGPP is converted to ent-kaurene by cyclization steps catalyzed by copalyl diphosphate synthase (CPPS) and kaurene synthase (KS). Ent-kaurene is then transported to the endoplasmic reticulum and oxidized by KO (P450 monooxygenase) enzyme to generate ent-kaurene acid (ent-KA). The ent-KA is the last common substrate for the SvGls and GA synthesis pathway. The hydroxylation of ent-KA at the C7 position favors the generation of GA, whereas the hydroxylation reaction at the C13 position leads to the formation of steviol (ent-13-hydroxy kaurenoic acid), which is catalyzed by kaurenoic acid 13-hydroxylase (KAH). This is one of the crucial steps involved in the SvGl (Figure 1).

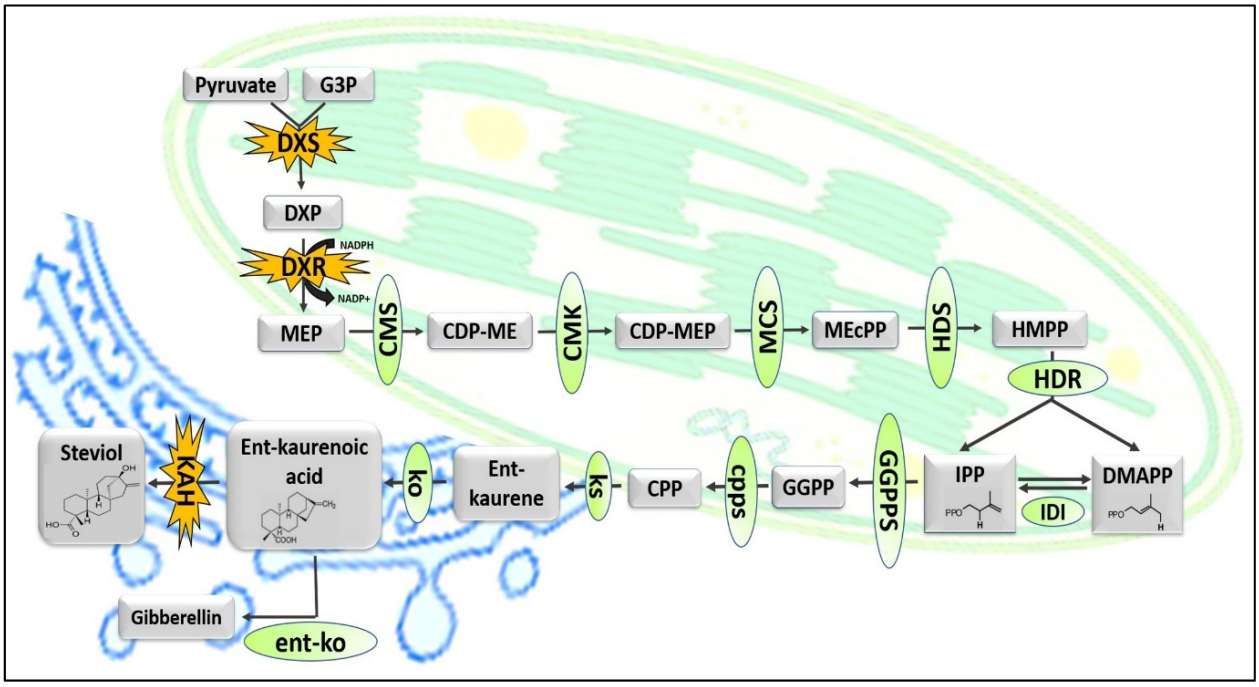


Figure 1. Schematic depiction of the biosynthesis of steviol glycosides by MEP pathway outlining the important enzymes involved in the pathway. G3P = Glyceraldehyde 3-phosphate; DXS = 1-deoxy-D-Xylulose 5-Phosphate; DXR = 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP = 2-C-Methyl-D-erythritol 4-phosphate; CMS = CDP-ME synthase; CDP-ME = methylerythritol cytidyl diphosphate; CMK = CDP-ME kinase; CDP-MEP = 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate; MCS = 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEcPP = 2-C-methyl-D-erythritol-2;4-cyclodiphosphate; HDS = 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HMPP = 4-hydroxy-3-methyl-butenyl 1-diphosphate; HDR = 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPP = Isopentyl diphosphate; DMAPP = dimethylallyl diphosphate; GGPP = geranylgeranyl diphosphate; GGPPS = GGPP synthase; CPPS = copalyl diphosphate synthase; CPP = ent-copalyl diphosphate; KS = ent-kaurene synthase; KO = kaurene 19-oxidase; ent-KO = ent kaurene 19-oxidase.

The final stage is the series of glycosylation, which takes place in the cytosol catalyzed by the family of UDP-dependent glycosyltransferases (UGTs). The glycosylation starts at the C13 position, and then at the C19 position, UGTs transfer the sugar moiety from the activated donor to the acceptor molecule. Different forms and different numbers of sugar molecules produce diverse SGs with varying properties. Four main UGTs which are involved in the formation of SvGls are UGT85C2, UGT74G1, UGT76G1, and UGT91D2, which are then transferred to the vacuoles [21]. It was suggested that UGT85C2 is the key

regulator for the synthesis of steviolmonoside, which is then glycosylated to stevioside. UGT91D2 is deciphered to play a major role in the synthesis of different rebaudiosides; in particular, it catalyzes the synthesis of Reb D from Reb A. Reb M, the newly popular sweetener, is also actively generated by three different crossways, with Reb A being the main backbone for its biosynthesis. Recently, Reb T, Reb U, and Reb Q were extracted from the leaves of stevia, although the sweetness concentration and their characterization are not well understood [22]. The detailed formation of different rebaudiosides and steviosides by different UGTs are represented in Figure 2.

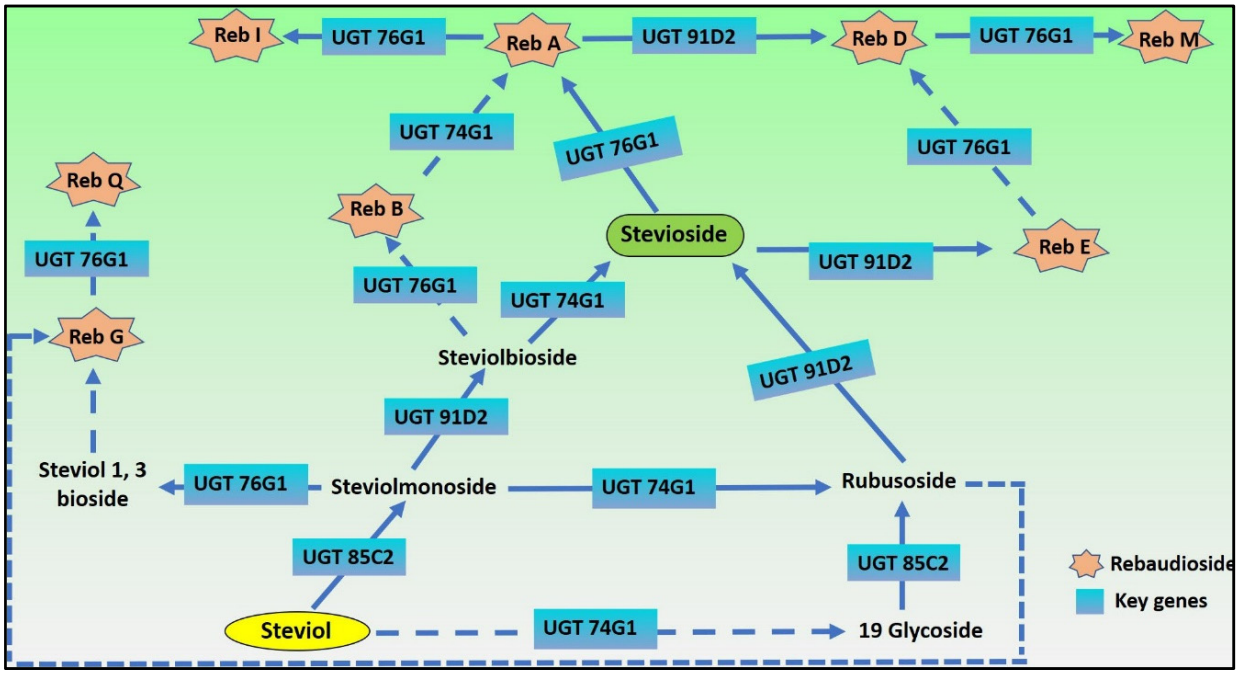


Figure 2. Glycosylation steps involved in the synthesis of steviol glycosides. Reb = Rebaudioside; UGT = UDP-dependent glycosyltransferases.

Most of the enzymes involved in the SvGls biosynthesis have been discovered and identified, and each enzyme may be the limiting factor of the biosynthesis pathway. However, it is suggested to explore novel genes that may be involved in biosynthesis of known (Reb D, Reb M, Reb A) and other unidentified sweeter steviosides. Furthermore, scientists may utilize metabolic engineering techniques to characterize and alter the gene expression of these enzymes to increase the production of specific SvGls.

4. Physical and Chemical Treatments to Improve SvGls Production

Plants can be impacted by a variety of environmental stresses, such as extremes of temperature, drought, alkalinity, salinity, UV stress, and pathogen infection [23]. Elicitation has been widely used to increase secondary metabolite production or to induce de novo synthesis in in vitro plant cell cultures. A number of elicitors have been used by researchers to enhance secondary metabolite production in plant cells, tissues, and organ cultures. UV-irradiation, high light, wounding, nutrient deficiencies, temperature, and herbicide treatment, are all known to increase the accumulation of phenylpropanoids. Nutrient stress has a significant impact on phenolic levels in plant tissues [24]. A lack of potassium, sulfur, and magnesium has also been linked to an increase in phenolic concentrations. Low iron levels can cause increased phenolic acid release from roots. Calcium levels have been linked to plant responses to a variety of abiotic stresses such as cold, drought, and salinity. Certain genes have been shown to increase in expression in response to reactive oxygen species, cold temperature, high temperature, and osmotic stress. Salt stress usually causes both ionic and osmotic stress in plants, resulting in the accumulation or decrease in specific

secondary metabolites. Likewise, different approaches used to boost steviol production have been described in details in the below sub-sections.

4.1. Alteration in Light and Photoperiod

Light, in general, has a positive effect on plants growth and development. Studies have shown that high light intensity increases the levels of stevioside and rebaudioside A and, thus, alters the production of SvGls. High levels of SvGls were also confirmed by analyzing the gene expression of *SrKA13H*, *SrUGT74G1*, and *SrUGT76G1*, which increased at higher light intensity [25]. The stevia plant is a short-day plant with a critical daylight requirement of 11–12 h to induce flowering. However, supplementing short night interruptions (NI) with low-intensity LED shortens the dark period and, thus, has been shown to have a negative effect on flowering. Delaying the flowering leads to an increase in vegetative growth and biomass of the plant, which is useful as steviol accumulation occurs in a tissue-specific manner in stevia. NI has been shown to increase the yield of harvesting of leaves 4–5 times annually and increase the production of stevioside and Reb A by 21% to 24% (kg ha^{-1}) [26]. Different types of LED also affect plant growth and secondary metabolite production. A study on the red/far-red LED and blue LED depicted that R/FR 1.22 and blue light treatment increases the transcripts level of *UGT85C2* and, hence, increases the SGs content in stevia plant grown in a controlled growth chamber [27]. Recently a combined approach for growing the micro propagated plants with high biomass in less time and enhancing the SvGls concentration was developed using RITA (Recipient for Automated Temporary Immersion System) and high-red LED. The findings reported an increase in biomass and gene expression of *ent-KO*, *ent-KS*, *ent-KAH13*, *UGT85C2*, *UGT74G1*, and *UGT76G1* with increases in stevioside and Reb A of up to 37.15% and 22.99%, respectively [28].

The possible explanation for the enhanced biosynthesis of secondary metabolites under the influence of LEDs may be the activation of photoreceptors, which activate signaling pathways and cause changes in gene expression. The light-absorbing properties are defined by the interaction of a photoreceptor protein and a chromophore. Many studies have suggested the potential use of LED and photoperiod, although the use of light to increase SvGls is still controversial, and further studies need to be conducted for its effect on SvGls synthesis.

4.2. Salt Stress to Enhance SvGls Production

Soil salinity poses a major constraint affecting plant growth, energy metabolism, and metabolites production. Different salt concentration induces different stress conditions, which accelerates varying response in plants. An amount of 30 mM NaCl (low salinity) concentration is shown to increase steviol concentration, and it is also suggested that the stevia plant is a moderate NaCl tolerant plant [29]. Several researchers have utilized elicitors to induce defense mechanisms in plants; for instance, NaCl levels of 50 mM and 150 mM along with 0.4 g/L chitosan increased stevioside and Reb A synthesis and produced a stress-tolerant plant [30]. The use of chitosan helps to induce a stress resistance mechanism that helped stevia plants to withstand high-salinity stress. These studies show the significant effect of salt stress on steviol production. However, many such experiments need to be performed to discover the optimum concentration of stress induction that can enhance high SvGls production in stevia plants.

Secondary metabolite production by a plant species is influenced by growth conditions and physiology and, to a large extent, by the differential impact of environmental growth conditions on metabolic pathways involved in their biosynthesis. Thus, several efforts have been made to enhance the secondary metabolite in several plants [31]. This resulted in the use of a significant number of elicitors of biotic and abiotic origin in improving metabolite production in *Stevia rebaudiana* via in vivo and in vitro growth condition manipulation, and increasing evidence from study results has established the role of oxidative stress defense response in secondary metabolite production in *Stevia rebaudiana* plants.

4.3. Elicitors to Enhance Synthesis and Gene Expression of SvGls

Elicitors such as hydrogen peroxide (H_2O_2), chitosan, methyl jasmonate (MeJA), and salicylic acid have shown improved SvGls (Reb A) ratios in the field. The foliar application of 20 mg/L chitosan and MeJA can increase the gene expression of *UGT85C2*, *UGT76G1*, and *KO* by 14–15%, respectively, while a higher concentration of yeast extract is required to increase the expression of *UGT85C2*, and *UGT76G1* genes [32]. Uptake of organic fertilizer enriched with nutrients in stevia plants also increases the SvGls synthesis, in particular, Stevioside and Reb A, Reb C [33]. Another study elucidated the application of NO_3^- on the increased SvGls synthesis in stevia leaves, although the nitrogen-induced accelerated SvGls synthesis mechanism needs to be explored further for its application in gene editing approaches [34]. Elicitors such as salicylic acid are observed to complement microelements (Fe, Zn)-enriched exogenous application on the synthesis of SvGls. Although many elicitors function by sending signals to the recipient, it seems that hormones act indirectly on gene expression and eventually biosynthesis of SvGls. However, the exact mechanisms remain unclear and require further investigations. A summary of the various conventional approaches involving physical and chemical processes used to enhance SvGls production in *S. rebaudiana* is represented in Table 1.

Table 1. Outline of the conventional (physical and chemical) approaches to enhance steviol glycosides concentration in *S. rebaudiana*.

Technique	Methodology	Target Metabolite	Output	References
Elicitors in field	H_2O_2, SA, chitosan	Stevioside and Reb A	Salicylic acid (0.1 mM) showed a significantly improved ratio of SvGls and Reb A	Vázquez-Hernández et al., 2018 [35]
Organic fertilizer	Nitrogen, calcium, magnesium and sulfur	Stevioside and Reb A, Reb C	Increase in the production of Stevioside and Reb A, Reb C	Díaz-Gutiérrez et al., 2020 [33]
Elicitors foliar application	Chitosan, MeJA, yeast extract	Steviol glycosides	Increased expression of UGTs involved in SvGls synthesis	Rasouli et al., 2018 [32]
Leaf infiltration	Daminozide (DAM) and NAA, GA3	Steviol glycosides	Increased transcriptional levels of *KO*, *UGT85C2*, and *UGT76G1*	Yoneda et al., 2018 [36]
Microelements application	SA with Fe, Zn	Stevioside and Rebaudioside	Increased concentration of Reb A, B, C, steviosides, Dulcoside A	Omidi et al., 2019 [37]
Stress and elicitor in field	NaCl stress and chitosan	Stevioside and Rebaudioside	Increased tolerance to stress and SvGls production	Gerami et al., 2020 [30]
Stress in field	NaCl	Steviol glycosides	30 mM of NaCl caused an increase in SvGls synthesis	Shahverdi et al., 2019 [29]

5. Breeding Approaches to Enhance Steviol Production

The primary goal of stevia breeding is the genetic improvement of existing stevia varieties with the development of varieties with a greater number of leaves, higher yield of SvGls and greater resistance to biotic and abiotic stress. The main traits desired to achieve a high success rate in stevia breeding are: high leaf yield per unit area, high content of specific SvGls (RebA, RebD, Reb M) in leaves, high adaptability to a wide range of climatic conditions and self-compatibility. Several patents have been registered for newly developed stevia varieties with enhanced traits desirable for steviol synthesis [8].

The conventional breeding approach involves two major steps: selection of genotypes and intercrossing among the genotypes with desirable traits. In the beginning, countries such as Japan, China, and Korea utilized the marker-assisted selection scheme (MAS) and reported success in their breeding programs by developing varieties with enhanced SvGls

and leaf yields. Some of these varieties were very high yielding but were limited for commercialization due to self-incompatibility [8,38]. Additionally, some findings suggested that recurrent phenotypic selection was more effective than MAS in improving quantitatively inherited characteristics in stevia—a cross-pollinated species characterized by high differentiation of a given trait within the population [39]. Further, synthetic cultivars were developed by intercrossing clones or sibbed lines derived from a breeding population through multiple cycles of recurrent selection. Synthetic varieties such as AC Black Bird and ATCC Accession No. PTA-444 are a few varieties developed with 2.5 times improved RebA-to-stevioside ratio compared to traditional stevia plants. However, these varieties also suffered from the loss of self-fertility [8].

Recently mutagenesis is the innovative approach that has brought technical and scientific progress in stevia breeding. Ethyl methanesulfonate (EMS), gamma radiation, and neutron beams are the commonly used physical and chemical inductions used to create an alteration in DNA. The random mutagenesis does not affect the germination growth of the seedlings and has shown a 1.5–2 fold increase in RebA content in comparison to the control plants [12]. The finding suggests that the increased SvGls content was associated with the increased expression of UGT74G1 induced by EMA and gamma radiations.

Characters of interest can only be improved through mutation breeding if the population's variability for the character in question is low. Because the leaves are the most valuable part of this crop, mutation breeding can play a significant role in stevia improvement.

6. Biotechnological Approaches to Increase Steviol Glycosides Production

Biotechnology techniques have provided wide possibilities and novel finding to enhance the synthesis of SvGls in in vitro and in vivo conditions.

6.1. Induction of Polyploidy

Induced polyploidy serves as an efficient approach to utilizing the genetic potential of cells. The treatment of explants with suitable antimitotic agents leads to an increase in biomass and enhances secondary metabolite production. The exposure time and colchicine concentration have a significant effect on the leaf size and production levels of secondary metabolites in the stevia plant [40]. Colchicine-induced ploidy in stevia plants is observed to significantly increase stem thickness, leaf thickness, leaf area, and stevioside content to 13.50% [41,42]. With the increasing popularity of sweeter SvGls, tetraploid stevia developed by induced polyploidy in seeds produced increased Reb A and stevioside content in the stevia plant. A recent comparative morphological and transcriptomic study on autotetraploid stevia and its diploid depicted a 1.27-fold increase in SvGls in tetraploid stevia [43,44]. The tetraploid stevia generated from stevia can further be used for micropropagation or selective breeding for increasing the SvGls content in stevia plants. However, these are only a few studies conducted to analyze the polyploidy effect, and further detailed studies and possible effects need to be explored.

6.2. Conventional Propagation and In Vitro Culture Technique

Propagation in *Stevia rebaudiana* has proved to be advantageous in overcoming the obstacles of self-incompatibility and less seed viability. Many in vitro propagations studies have been conducted to achieve genetically identical stevia plants with uniform levels of SvGls. Recent studies have shown a higher success rate of 98–100% and increased survival rate of in vitro-propagated plants [45,46]. Additionally, several elicitors and stress environment (salt stress) have proved to be beneficial for increased SvGls synthesis in a fast and cost-effective manner [47–50]. The possible explanation for this may be the stress-induced changes in the membrane structure, chlorophyll content, and enzyme activity which influence the production of primary and secondary metabolites in the plants.

The poor seed viability and low germination rate are major constraints for conventional propagation of the stevia plant. Moreover, cross-pollination makes it difficult to raise an elite population with uniformly high SvGls content [51]. The role of vegetative propagation

is also limited because specific habitat conditions are required to grow the plants, in addition to a low soil acclimatization rate.

In vitro multiplication of *S. rebaudiana* elite genotypes is a promising alternative to meet the increasing industrial demand for its use as a food additive and for therapeutic purposes. Supplementing the growth hormone and engineered nanoparticles such as Fe, ZnO, and CuO to the different stevia explants has shown a positive impact on the development of callus and cell culture for the production of SvGls [52–59]. The combination of the growth hormones is vital for cell proliferation of cells from *Stevia rebaudiana*, while the production of the SvGls depends on the genotype utilized for the explant [60]. Studies to understand the organogenesis mechanism have deciphered the role of differentiation of mature chloroplast crucial for SvGls maximum output [4].

Therefore, it may be inferred that *Stevia rebaudiana* plants generated by the micropropagation showed no negative effect on steviol production, although some controversies exist, claiming a 4–5-fold decrease in steviol production. Considering time and cost management, micropropagation of stevia plants seems to be an effective approach for the mass production of steviol glycosides. However, the poor seed viability and low germination rate are major constraints for the conventional propagation of the stevia plant. Moreover, cross-pollination makes it difficult to raise an elite population with uniformly high SvGls content [51]. Thus, in vitro culture seems to be the potential strategy to exponentially enhance the SvGls production. Moreover, the mass production of adventitious root for SvGls synthesis under the influence of nutrient supplements [53] and application of melatonin (MEL) [61] as a plant growth supplement and stress inducer (under saline conditions) are unique and very advantageous approaches for bulk production of SvGls. Additionally, the reverse strategy to inhibit the GA synthesis [62] may be a promising approach to enhance SvGls levels. However, further work in this area will establish the optimum growth condition for growing the stevia plants under several stress conditions. These findings open the way to utilize enhanced production of SvGls in stevia tissue cultured plants for the nutraceutical industry. Moreover, it provides a new platform for the study of metabolic pathways involved with the interaction of nanoparticles and micro-propagated plantlets.

6.3. Transformation Technique to Enhance SvGls Production

Altering the gene activity via gene manipulation is the most powerful technique to elucidate the function of genes involved in the biosynthesis of SvGls. However, the expression of a manipulated gene requires efficient stevia tissue culture and transformation system. Earlier discussed studies report the successful tissue culture methodologies using various explants. Recently, a few studies were conducted to establish a stable transformation system in stevia using *Agrobacterium*-mediated gene transfer. The studies targeted the SvGls synthesis enzymes by using RNAi constructs and transient expression system. Following this, *Agrobacterium*-mediated transformation system of stevia leaf disc using the GUS reporter gene was introduced. The callus was successfully generated, although there was no report for whole plant regeneration [63]. Several studies overexpressed key regulatory genes such as *SrDXS1*, *SrKAH* % [64] and isoforms of *SrUGT76G1* [22] and were found to be effective in enhancing the SvGls production without compromising the development of stevia plants, thus suggesting the potential use of a transformation system and mass production of stevia plants with enhanced desirable SvGls.

Recently, the application of hairy root culture has also been employed in stevia plants by transforming it by *A. rhizogens* for induction of hairy root [21]. More studies and clarification need to be made to utilize the technique of transformed hairy root culture. The study suggests the potential of hairy root culture for mass production and elucidation of the synthetic pathway by genetic manipulation of the genes. Table 2 depicts the various biotechnological strategies applied by several researchers to enhance SvGls production in *S. rebaudiana*.

Table 2. Overview of the biotechnological strategies used to enhance SvGls production in *S. rebaudiana*.

Technique	Methodology	Traits	Output	References
Ploidy induction	Seed + 0.2% colchicine (24 h) induce tetraploidy	Reb A	Reb A (2.84% dry weight)	Talei et al., 2020 [40]
Ploidy induction	Seed + 0.1% colchicine (24 h) induce tetraploidy	Stevioside Reb A	stevioside (2.5-fold) and Reb A (1.5-fold)	Zhang et al., 2018 [65]
Ploidy induction	Adventitious bud + 0.20% colchicine (12 h) induce tetraploidy	Stevioside	SvGls increased by 1.27-fold	Xiang et al., 2019 [43]
Ploidy induction	Axillary buds + 1% colchicine (3 days) induce tetraploidy	Stevioside	SvGls increased by 2.5-fold	Mahdi et al., 2018 [44]
Micropropagation with elicitors	WPM, MeJA, SA, and CHI	Stevioside	17.4 times stevioside production than control at 100 µM MeJA	Bayraktar et al., 2018 [47]
Micropropagation with elicitors	Glutamine	Stevioside	Highest amount of stevioside (22.74) and Reb (12.19) was seen under 2% glutamine	Esmaeili et al., 2018 [48]
Micropropagation with elicitors	Chitosan, yeast extract, MeJA	Stevioside and Reb A	MeJA 100 and CH 200 mg/L significantly enhanced the Reb A/ST ratio	Rasouli et al., 2021 [49]
Micropropagation with salinity stress	NaCl	Steviol glycosides	Upregulation of *CMS*, *CMK*, *HDR*, and *UGT76G1* involved in SvGl synthesis	Lucho et al., 2019 [50]
Seed culture	Agar gel, NaCl	Stevioside and Reb A	Upregulation *SrIDI*, *SrCPPS1* involved in SvGls synthesis	Simlat et al., 2020 [61]
Callus and cell suspension culture	growth regulators	Major and minor SvGls	Ten times higher SvGls production	Bondarev et al., 2019 [60]
Adventitious root culture	GA3, NAA	Stevioside, Reb A, dulcoside-A	2.0 mg/L GA3 increased the production of Stevioside and Reb A, dulcoside-A in roots	Ahmad et al., 2020 [53]
Lateral bud culture with elicitors	SA, yeast extract	Stevioside, Reb	increase in steviol glycosides content overexpression *KA13H*, *UGT74G1*, *UGT76G1*, *UGT85C2*	Mehravaran et al., 2021 [56]
Axillary buds with elicitor	Mannitol	Stevioside, Reb	Highest stevioside produced at 20 g/L and highest rebaudioside produced at 30 g/L; increased expression of *UGT76G1*, *UGT74G1*, *KS*, *KO*	Ghaheri et al., 2019 [57]
Shoot culture with nanoparticles	Engineered zinc oxide (ZnO)	Stevioside, Reb A	Increase in steviol glycosides production (88.21 mg g^{-1} DW)	Javed et al., 2017 [52]
Callus culture with nanoparticles	Silver nanoparticles, NAA, BA	Stevioside	Enhanced production of stevioside by 67%	Golkar et al., 2019 [55]
Root culture with nanoparticles	ZnO and CuO	Steviol glycosides	Increased rebaudioside A (4.42 and 4.44) and stevioside (1.28 and 1.96)	Ahmad et al., 2020 [59]
Nodal explants with nanoparticles	Fe nanoparticle	Stevioside, Reb A	Stevioside: 4.2 ± 0.058 mg/g (DW) and rebaudioside A: 4.9 ± 0.068 mg/g DW	Khan et al., 2020 [54]
Agrobacterium-mediated transformation of axillary shoots	Overexpression of UGT76G1	Reb A, Reb B, Reb C, Reb D and Reb G	Reb A production was increased twice that of stevioside	Zhang et al., 2020 [22]
Agrobacterium-mediated transformation	overexpressed the SrDXS1 and SrKAH	Steviol Glycosides	Transgenic lines SvGls were enhanced by up to 42–54% and 67–88%	Zheng et al., 2019 [64]
Agrobacterium-mediated foliar explants transformation	35S CMV promoter	Stevioside, Reb A	1.4- and 1.5-fold production increase in stevioside and Reb A	Sanchéz-Cordova et al., 2019 [66]
Transformation with *A. rhizogenes* and hairy roots production	Transformation under light stress and osmotic stress	Reb, stevioside, steviolbioside and Reb F	The concentration of rebaudioside increased on hairy root under oxidative stress	Libik-Konieczny et al., 2021 [21]

6.4. Transcriptomic Profiling of Genes Involved in Steviol Glycosides Synthesis

Different plant tissues and organs reveal differential gene expression patterns that vary in time and space. These distinctions are critical in research on functional gene mining, development of gene regulatory networks, determination of secondary metabolites, resistance genes, and genetic diversity in medicinal and industrial plants. *S. rebaudiana* is a good

source of zero-calorie sugar, and the increasing popularization and continuous demand of SvGls makes it important to decipher the molecular mechanism underlying SvGls synthesis. Over the years, constant efforts have been made to identify and characterize the key genes and regulatory networks determining SvGls synthesis in *Arabidopsis* and *S. rebaudiana*. Table 3 represents the cloning and characterization of genes encoding regulatory enzymes involved in the synthesis of SvGls.

Table 3. Overview of the characterization and expression analysis of the genes involved in steviol glycosides biosynthesis pathway.

Target Gene	Characterization of Gene	Expression Assay	Reference
DXS	Characterized in Mentha (peppermint)	*E. coli*	Lange et al., 1998 [67]
DXS	Characterized in *Stevia rebaudiana*	*E. coli strain MC4100 dxs::CAT*	Totte et al., 2003 [68]
DXR	Cloned from *Arabidopsis* and *Mentha* (peppermint)	*E. coli*	Lange and Croteau, 1999 [69]
DXR	Characterized in *S. rebaudiana*	*E. coli strain MC4100 dxr::TET*	Totte et al., 2003 [68]
MCS	Characterization in *Arabidopsis thaliana*	*E. coli ispF* mutant strain *EB370*	Hsieh et al., 2006 [70]
CMS	Characterization in *A. thaliana*	*E. coli XL-1 Blue*; *M15 (pREP4)*	Rohdich et al., 2000 [71]
GCPE	Characterization in *A. thaliana*	*E. coli* strain *EcAB3-3*	Querol et al., 2002 [72]
CMK	Characterization in tomato	*E. coli XL-1 Blue*	Rohdich et al., 2000b [73]
CPS, *KS*	Characterized in *S. rebaudiana*	Recombinant CPS and KS proteins expression by pET30a and pET30b expression vector	Richmann et al., 1999 [74]
CPS, *KS*	Characterized in *S. rebaudiana*	*E. coli XL-1 Blue*	Richmann et al., 1999 [74]
KO	Not characterized; Cloned and expressed in yeast	*E. coli XL1-Blue MRF* Functional assay by epYES2/NT yeast expression vector	Humphrey et al., 2006 [75]
KAH	KAH not characterized KAH homologs CYP714A2 and CYP716 characterized in *A. thaliana*	Expression assay in yeast cells	Nomura et al., 2013 [76]
UGT85C2, *UGT74G1*, *UGT76G1*	Characterized in *S. rebaudiana*	Expressed in BL21-CodonPlus (DE3) *E. coli*	Brandle and Telmer, 2007 [77]
UGT76G1	Characterized in *S. rebaudiana*	*Agrobacterium*-mediated transient expression in Nicotiana	Petit et al., 2020 [78]
UGT91D2	Characterized in *S. rebaudiana*	*E. coli BL21 (DE3)* pXL17/pXL13	Wang et al., 2016 [79]

Proteomics, genomics, and transcriptomics have emerged as popular approaches in the post-genomic era. Transcriptomics is currently the most extensively researched and applied discipline, allowing for a deep understanding of how genes are expressed and related. With the recent advances in sequencing technology, transcriptome research tools have evolved from the traditional chip hybridization platform to RNA sequencing technology (RNA-Seq) [80]. Transcriptomics is proving to be one of the most significant approaches to identify the genes regulating the steviol production [81]. Initial studies of RNA-Seq in stevia plants reported annotation of 80,160 unigenes of which 143 UGT unigenes were identified, suggesting their role in SvGls biosynthesis.

The transcriptomic profiling revealed the high expression levels of *DXS*, *MDS*, *HDS*, *KO*, *UGT85C2* and *UGT76G1*, while *GGDPS* depicted low expression levels [82]. Further global transcriptomic analysis deciphered 41,262 de novo transcripts involved in steviol and gibberellin synthesis. Differentially expressed genes (DEGs) such as *DXS*, *HMGR*, *KA13H* genes, *WRKY*, *MYB*, *NAC TFs* gene regulators, 24 *CYPs* and 45 *UGTs* indicated their employment in the early developmental phases of SvGls and gibberellin biosynthesis [83]. High expression of these genes in leaf tissue vegetative growth compared to bud phase and

flowering phase indicates that as the plant develops and proceeds towards the flowering stage, the accumulation or production of SvGls exponentially decreases.

At present, the market demands the availability of sweeter SvGls, and thus, a comparative transcriptomic study on genotypes with varying levels of stevioside, Reb A, Reb D and Reb M deciphered DEGs including 7 UGTs and 76 transcription factors representing varying coherence of Reb D and Reb M synthesis in stevia leaves. This study provided a better analysis of a regulatory network of these rebaudiosides in stevia [84]. Various approaches such as the use of elicitors and stress conditions are generated to study their effect on steviol synthesis. Likewise, the nitrogen deficit conditions induced high levels of SvGls synthesis in stevia plants. The transcriptomic analysis also confirmed the output by identifying DEGs involved in the upregulation of steviol synthesis. The nitrogen deficiency induces the change in carbon metabolism flux and certain transcription factors which leads to enhanced production of SvGls [85]. Recently, transcriptomic profiling was utilized to identify and design 360 SSR belonging to the UDP-Glycosyltransferase protein family and Deoxyxylulose-5-phosphate synthase involved in the steviol biosynthesis. The SSR generated through this study is a stepping stone for the molecular breeding of stevia to generate stevia varieties with enhanced SvGls [86].

However, more studies are required to determine the DEGs and simultaneously develop SNP or SSR for the development of superior stevia varieties through a breeding program. Moreover, the identification of the underlying genes by transcriptome profiling will enable its further utilization of metabolic engineering or genetic engineering to enhance primary or secondary metabolite production.

6.5. Metabolic Engineering of Microorganisms

Most of the SvGls identified so far are extracted from *Stevia rebaudiana*. However, the extraction rates of SvGls from the stevia plant are quite low resulting in insufficient supply and high cost. Thus, nowadays, metabolic engineering is being utilized for heterologous production of SvGls in metabolic engineered microorganisms [87]. This had led to tremendous production and commercialization of SvGls in the global market. Metabolic engineering utilizes the methodologies of enzyme engineering, modular synthesis of enzymes, and regulating the gene expression to enhance the production of enzymes/biocatalyst involved in steviol synthesis.

Earlier studies reported the construction of yeast cells for de novo synthesis of SvGls, although limited success was attained by using this approach. Recently, researchers have constructed the de novo synthesis of SvGls by modulating the functional expression [79] and overexpressing and altering the 5′ untranslated region (UTR) of key enzymes and ratio of cellular NADPH/NADP+ [88] in the *E. coli* model system. The findings are indicative that modulating the redox potential may increase the production of SvGls in *E. coli*.

S. cerevisiae proved to be another suitable heterologous system for the production of terpenoids by using simple glucose to form steviol for de novo synthesis of SvGls [89]. Specifically, screening and utilizing perfect CYP-CPR homologous pairs seems to be a promising approach for enhancing the SvGls production. In the efforts to increase SvGls production, some researchers have engineered photosynthetic cyanobacteria to produce ent-kaurenoic acid directly from CO2. Engineered *Synechoccous elongatus* strain was used to express the cytochrome P450 enzymes CYP79A1 and CYP71E1, which enabled the synthesis of ent-KA at 2.9 ± 0.01 mg L^{-1} from CO2 [90].

Glucosyl transferases, in particular, members of the GT1 family, are crucial for glycosylation in the production of the desired rebaudiosides and stevioside. GT1 members use UDP-activated sugars as glycosyl donors, and most of them belong to UDP-dependent glycosyltransferases (UGTs). A whole-cell biocatalysis system was used to express UGT76G1 by constitutive expression system in *S.cerevisiae.* The study deciphers that optimizing the whole-cell response parameters (pH, cell permeability, temperature, glucose concentration) can enhance Reb A. Recently, researchers have developed the pathway for Reb D synthesis by coupling UDP-glucosyltransferase UGTSL2 from *Solanum lycopersicum* and sucrose

synthase StSUS1 from *Solanum tuberosum* in *E. coli* [91]. The recent advancement to earlier reports is the establishment of a multi-enzyme reaction system to enhance the production of sweeter steviols such as *Reb D* [92]. Recent approaches to increase the production of SvGls by using different substrates, strains of microorganisms and fermentation methods are discussed in Table 4.

Table 4. Overview of the summarized table depicting the developments made in the metabolic engineering for the production of steviol glycosides.

Target Enzyme	Strain Used	Methodology	Production Rate	Reference
Ent-kaurene	*E. coli BL21 (DE3)*	IPTG (0.02 mM) and fermentation temperature (30 °C), the maximum yield of ent-kaurene was improved	2.16 mg L^{-1} to 194.12 mg L^{-1} (shake flask) and 1.872 g L^{-1} (in 5-l bioreactor)	Wang et al., 2016 [79]
Ent-KA	*E. coli BL21 (SSY10)*	Enhanced expression of *KO-Sr* and optimized fermentation temperature (22 °C) and IPTG (0.1 mM) concentration	100.23 mg/L	Wang et al., 2016 [79]
Steviol	*E. coli BL21 (SSY10)*	Replaced stevia derived KAH by engineering N-terminus of *A. thaliana CYP714A2* to *17αTR29CYP714A2*	15.47 mg/L	Wang et al., 2016 [79]
Reb A	*E. coli BL21 (SSY10)*	UGT module *UGT85C2/ UGT91D2w/UGT74G1/UGT76G1* incorporated into *17αTR29CYP714A2*	10.03 mg/L	Wang et al., 2016 [79]
Ent-KA	*E. coli BL21*	5′ UTR of *GGPPS*, *CPPS*, and *KS* engineered and expressed	623.6 ± 3 mg/L (batch)	Moon et al., 2020 [88]
Ent-KA	*E. coli BL21*	Overexpressing engineered 5′-UTR, N-terminus of *A. thaliana KO* and increasing the cellular ratio of NADPH/NADP+	50.7 ± 9.8 mg/L (batch)	Moon et al., 2020 [88]
Steviol	*E. coli BL21*	5′UTR engineered trCYP714A2 and N-terminus modified (UTRCYP714A2-ATCPR2) fusion protein overexpressed	38.4 ± 1.7 mg/L (batch)	Moon et al., 2020 [88]
Ent-KA	*S. cerevisiae*	Glucose used as a substrate, CYP-CPR combinations optimized and optimal KO-KAH-CPR combinations identified	<90 mg/L	Gold et al., 2018 [89]
Ent-KA	*Synechococcus elongatus PCC 7942*	Engineered cyanobacteria to produce ent-kaurenoic acid from CO2.	2.9 ± 0.01 mg/L	Ko and Woo 2020 [90]
Reb A	*S. cerevisiae*	Whole-cell biocatalyst used for expression of *UGT76G1*, and whole-cell parameters setup for cell permeability, temperature, pH, citrate, and Mg^{2+} concentrations, and glucose supply.	1160.5 mg/L (substrate is added 2 g/L stevioside)	Li et al., 2016 [93]
Reb D	*E. coli BL21*	Coupling *UGTSL2* from *Solanum lycopersicum* and *StSUS1* from *Solanum tuberosum* to construct a SuSy-GT for overexpression of *RebD*	17.4 g/L (substrate is added 20 g/L Reb A)	Chen et al., 2018 [91]
Reb D	*E. coli BL21*	Established multi-enzyme reaction system with *UGT76G1*, *UGTSL2*, and *StSUS1*, and replaced wild-type *UGTSL2* with *Asn358phe* mutant	14.4 g/L (substrate is added 20 g/L stevioside)	Chen et al., 2020 [92]

Table 4. *Cont.*

Target Enzyme	Strain Used	Methodology	Production Rate	Reference
Reb D	*Pichia pastoris*	One-pot synthesis utilized for heterologous expression of EUGT11 from *Oryza sativa* forming XE-3 transformant	Conversion rate reached 95.3%	Wang et al., 2020 [94]
Syevioside Reb A	*Pichia pastoris*	*TtbGal1* and *MtBgl3a* expressed in Pichia pastoris	Conversion of 34.6% (stevioside) and 25.6–35.6% (RebA)	Zerva et al., 2021 [95]
Reb A Reb M	*E. coli BL21*	Co-expression of endogenous prpD and malK in *E. coli* improved the expression of *Smt3-UGT76G1*.	4.8 g/L (RebA) 1.8 g/L (RebM)	Shu et al., 2020 [96]

These studies report the potential use of metabolic engineering for large-scale production of sweeter Rebaudiosides by expression of key enzymes in a heterologous system. Protein engineering has become a powerful tool for the development of metabolic engineering. It enables the increase in protein activity by modulating the catalytic activities and stability. The 3D design and de novo computational design of protein molecules are becoming more popular due to their important role in enzyme engineering and synthetic biology. Moreover, manipulating the microbial strains for the targeted output of enzymes will further increase the yield and desired quality SvGls.

7. Extraction and Purification Methods for SvGls Production

Dehydration and extraction are the two basic steps that determine the extraction rate of SvGls from the leaves of *Stevia rebaudiana*. The type of solvent and the manufacturing process have a large impact on the yield of its metabolites. Some other parameters which play a crucial role in the extraction process are the operating temperature (60–100 °C), exposure time (30 min to 24 h), devices, and materials (e.g., resins). The common dehydration or drying processes used are freeze-drying, vacuum drying, microwave drying, infra-red drying, sun-drying, and shade drying. The traditional methods of sun drying and shade drying are more prone to microbial contamination and have an adverse impact on their quality. In the case of stevia, convective and infra-red drying is suitable to increase its shelf life by restricting the growth of *Listeria innocua*. Thus, an effectively optimized drying condition is necessary to eliminate the adverse effect affecting the antimicrobial and antioxidant activity of the stevia extracts. Steviol glycosides are highly stable at normal temperature and pH. Both major and minor SvGls are thermally and hydrolytically stable at normal temperature and storage conditions. This characteristic of SvGls makes them highly desirable ingredients for the food industry.

The extraction efficiency of the SvGls and other metabolites is dependent on the type of solvent and technique used for the extraction process. Water is observed to be the best solvent for stevia phytoconstituent extraction. The extracts obtained from the mixture of propylene glycol and water in the ratio (4:1) produced a maximum concentration of phenols, and flavonoids [97]. The use of organic solvent preserves the antioxidant property of the extracts but may cause fibroblast irritation when used as a food additive or in cosmetics, probably due to cytotoxicity. Thus, recently, the concept of greener options for SvGls extraction has become more desirable. Gallo et al. [98] used a new solid–liquid extraction method employing a Naviglio extractor and water as solvent at room temperature. This method was effective in saving energy consumption as it yielded 1197.8 and 413.6 mg/L of Reb A and stevioside, respectively, in just 20 min. Extraction techniques along with temperature, extraction time, pressure, and particle size have a large influence on the extraction rate. Classical methods such as maceration and heat extraction are very lengthy, and thus, these methods are being eliminated. Various new approaches to improve extraction yields and purity of molecules have been proposed such as pressurized fluid extraction (PFE), microwave-assisted extraction (MAE), high voltage electrical discharge, pulsed electric

field, and ultrasound-assisted extraction (UAE), and chromatographic techniques. These methodologies are superior to conventional approaches, although their extraction yield is quite low, and they require a large number of solvents, which makes them economically non-feasible for practical applications of steviol extraction.

Currently, membrane-based technology is used for extraction of metabolites from fermented broth, and recovery from high-added-value molecules from the liquid extract is also the current trend in the field [99]. Recent reports deciphered 25–80% steviol glycosides extraction yield and purity depending on the type of membrane, operating parameters, and pre-treatment steps. The integrated membrane process depicts the highest extraction yield and purity and serves as a promising approach for the extraction and purification of SvGls [100]. However, the scale-up of the membrane-based extraction of SvGls is required to meet the increasing demand in the food and pharmaceutical industry.

8. Commercialization Status of Stevia

The flavor of sugar (sweetness) is highly desirable in several food products. Until the 20th century, sugar was considered irreplaceable due to its taste and sweetness. However, high consumption of sugar in everyday life has created some serious health concerns such as diabetes, obesity, and heart diseases. This has led to dependability on alternate sugar sources in particular aspartame, cyclamate, glycyrrhizin, saccharin, and steviol glycosides. These artificial sugars are known to be associated with increased health risks. Aspartame is the most widely used sweetener, and it may cause serious health issues, including cancer, Alzheimer's disease, and cardiovascular problems. Cyclamate forms a substance CHA, which was found to cause severe testicular damage. High doses of glycyrrhizin may cause pseudoaldosteronism, which may further lead to multi-organ failure in patients. Saccharin was found to cause liver disorders and bladder cancer. Earlier reports also suggested that stevia is carcinogenic and highly unsafe for consumption. Later, dedicated research on stevia proved its safe consumption without any health risks [101]. Therefore, stevia is now considered the best third-generation calorie natural sweetener.

Stevia and its glycoside's remarkable sweetening intensity (~300 times more than sucrose) have attracted the attention of various food and pharmaceutical industries. Steviol glycoside's high resistance to heat makes it an excellent choice as a sweetener for the baking and food industry. Its high absorption capacity makes it highly desirable for making bread and highly viscous food products. According to some studies, stevia has been shown to increase the shelf life of the bread by 5–7 days. Optimum stevia concentration increases the properties of bread yeast and improves bread quality [102]. Stevia products are low in glycemic index and thus beneficial for diabetes patients. These properties of stevia have widened its acceptability and adaptability in the food industry. The safe dosage of stevia is around 0.25–1 g for confectionery products and 25 mg for dairy products [103].

Stevia's use as a commercialized product has been debatable in several countries for years. Japan was the first country to commercialize stevia as a natural sweetener in early 1971. They have been using this product in various food products and cold beverages. Currently, Japan holds a significant share of the sweetener market. Eventually, stevia gained popularity in the US, and in 2008, the Food and Agriculture Organization and the World Health Organization's Joint Expert Committee on Food Additives (JECFA) approved the use of steviol glycosides. Later in 2008, stevia was also provided a GRAS (generally regarded as safe) certificate and was classified safe for consumption in the USA (FAO fact sheet, 2008) [104]. Following the trail, France approved the usage of Reb A in 2009. Finally, in the year 2011, SvGls were approved by the EFSA in the European Union EU. At present, stevia consumption and market are approved in most countries. According to the latest reports, China followed by Japan is the leading producer of stevia. A few countries such as Australia and New Zealand have generated guidelines for stevia usage in a range of food products. In Australia, unprocessed and crystal forms of SvGls are only allowed for commercial sale [105].

In 2007, in a joint venture, The Coca-Cola Company and Cargill commercialized Truvia, containing erythritol and Rebiana. Rebiana is known to produce a higher level of Reb A, which is much sweeter than other SvGls. Later, in 2013, Coca-Cola Life drink was introduced in New Zealand and was launched in several other countries where stevia consumption was approved [106]. Eventually, Pepsico and Purecircle announce their brand PureVia for a stevia-based sweetener and also released their drink containing the natural sweetener RebA [107]. In 2017, SweetGen commercialized BESTEVIA RebM and RebD with a high purity grade at the global market. It has been well received by many foods and beverage companies in Asia, the US, and Europe. Reb I molecule, produced by SweetGen, has recently obtained its no-objection certificate from FDA and GRAS and is ready to be commercially available in the near future (Reports, 2021). Recently, in 2020, Ingredion acquired a 75% stake in PureCircle, which is offering a Reb M sweetener extracted directly from stevia leaves. Reb M is a newly identified best-tasting sweetener, and it holds high value in the market. In another collaboration, Cargill and DSM are working on the large-scale development of metabolic engineered production of RebM (brand name: EverSweet). EverSweet is environmentally friendly since the carbon footprint of its bioconversion is 60% lower than other sources, and it requires 70% less land area [108].

The global natural sweetener market is exponentially increasing and is estimated to reach USD 3.8 billion by 2025 with a CAGR of 8.8%. The production volume of stevia is expected to reach 72,670.9 tonnes by 2028 with a growth rate of 6.7% [109]. Stevia market is divided into three categories: extract powder, liquid, and leaves. The stevia extract powder generated the highest revenue in 2021. It is popular because of its ease of handling and accurate dosage efficiency compared to liquid extract, thus being a key contributor in the global market. Based on the application, the stevia market is classified into beverages, bakeries, confectioneries, dairy products, and dietary supplements. Recent developments show that in 2018, S&W Seed Company expanded its agreement with an international consumer company to produce non-GMO stevia crops by breeding and targeting novel superior quality traits [110]. Likewise, in 2021, Tate & Lyle and Codexis strengthened their partnership to increase the current production rate of commercially available stevia-based sweeteners DOLCIA PRIMA® Allulose and TASTEVA® M [111]. In April 2021, another major influencer, Archer-Daniels-Midland Company, expanded its R&D sector in Singapore and it is expected to develop next-level on-trend and nutritious products to meet the current demand for zero-calorie food and beverages in Asian provinces [112]. Figure 3 represents the summary of the different approaches and strategies utilized so far by the researchers to enhance SvGls production and marketability.

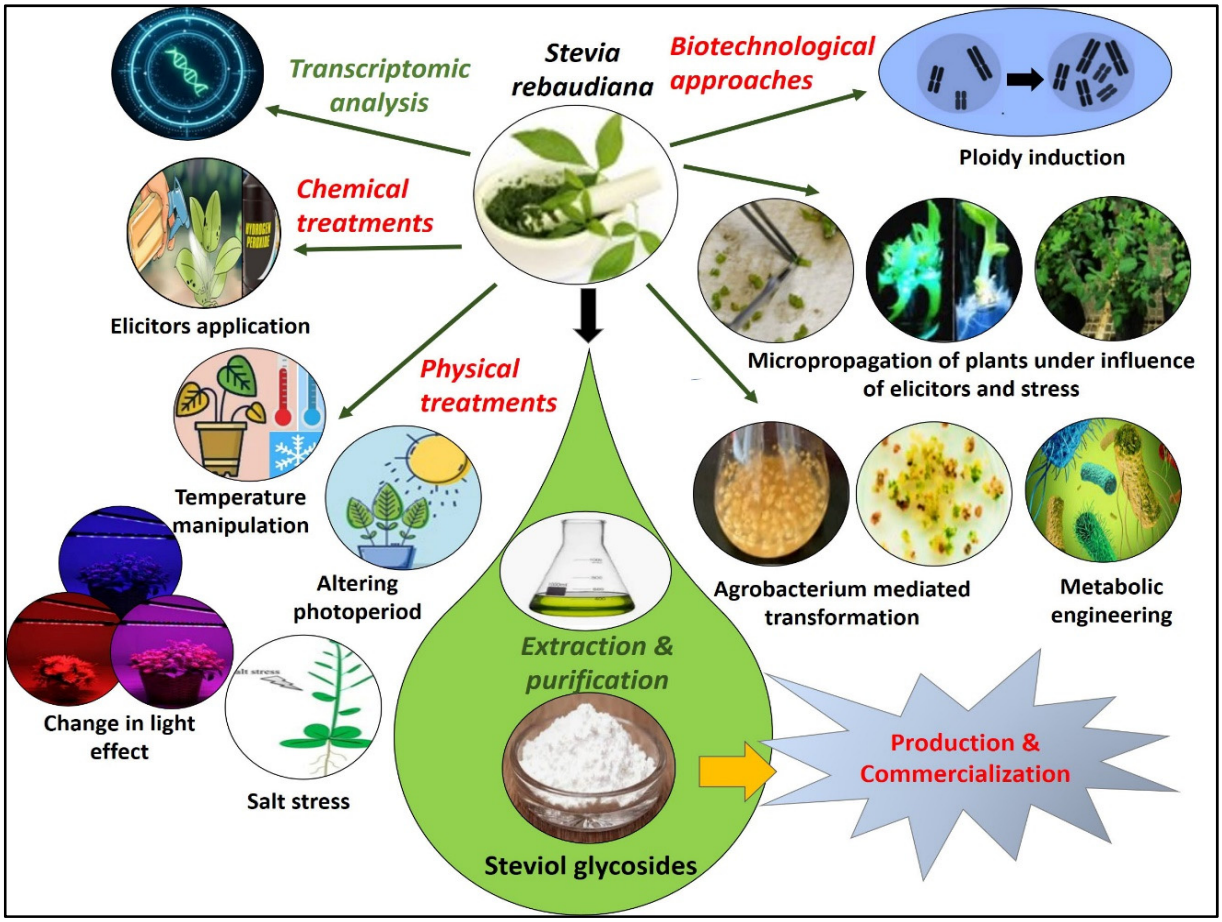


Figure 3. Pictorial representation of the various approaches employed to increase steviol glycosides synthesis. Libik et al. [21]; Zhao et al. [113]; Cox et al. [114].

9. Summaries

The current review represents an overview of the various advancements occurring in the elucidation of biosynthetic pathways and enhancement of SvGls production to meet the current demand. The natural sweetener steviol glycosides are chiefly produced from the leaves of *Stevia rebaudiana*. Stevia plants with a high leaf-to-stem ratio are highly desirable for achieving steviol's maximum extraction rate. SvGls are beneficial to human health, possessing anti-diabetic properties and high antioxidant activity, helping in lowering blood pressure, and foremost having a very similar sweet taste to sugar (sucrose). Developing methods to enhance steviol production is of high interest to the food and pharmaceutical industry. Stevia morphological behavior has set constraints in developing improved stevia variety by conventional breeding.

Various conventional and biotechnological approaches have been applied to enhance the synthesis of SvGls. Several plant tissue culture methodologies have been suggested to micro propagate stevia cuttings and stems for large-scale production of stevia plants. Advanced biotechnology practices such as callus culture, adventitious root culture along with the use of elicitors, transformation techniques, and metabolic engineering (using bacterial strains) showed positive effects in increasing the production of SvGls. Studies by Golkar et al. [55], Ahmad et al. [53] and Khan et al. [54] using nanoparticles with callus/nodal explant/root culture showed a significant increase of 60–70% in SvGls. Likewise, recent reports by Zheng et al. [64] and Wu et al. [22] showed that overexpressing key genes *UGT76G1*, *SrDXS1* and *SrKAH* involved in the biosynthesis pathway of steviols enhanced the SvGls concentration in the transgenic stevia leaves. These methodologies offer some promising results and expand the way to manipulate the experiments to improve SvGls pro-

duction. RNA-Seq methodology, utilized by some researchers to elucidate genes governing sweeter SvGls and the development of SSR markers, shows the potential of transcriptomic for its utilization in genetic engineering and stevia breeding [84]. However, detailed studies need to be conducted to characterize and understand to role of enzymes involved in the pathway. Moreover, the function of these key enzymes in plant physiology is also an area that must be explored for better insight into gene regulation. Gene transformation and bulk production of SvGls via metabolic engineering seem to be the future approaches that seem to be of particular importance.

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