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BIOLOGICAL TECHNOLOGY METHODS FOR METABOLIC ENGINEERING OF PLANT TERPENOID BIOSYNTHESIS

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ABSTRACT

The deliberate alteration of cellular metabolism to provide desired chemicals is known as metabolic engineering. Recombinant DNA technology allows for the manipulation of different species' metabolic processes. The groundbreaking focus on whole metabolic networks as opposed to isolated processes set the stage for metabolic engineering to develop into a separate branch of science. Originally interpreted as a compilation of instances from scientific and pharmaceutical studies, its true significance surfaced when a methodical strategy was required to substitute the haphazard mutagenesis selection procedure. Primary metabolites (lipids, carbohydrates, etc.), biofuels, polyamines, recombinant proteins, amino acids, secondary metabolites (alkaloids, terpenoids, flavonoids), polymers, and biotech crops are examples of significant industrial products. By upregulating these pathways, crops can become more nutritionally rich since many plant metabolites have significant positive effects on health and nutrition. In the human diet, phenolpropanoid and terpenoid molecules have crucial nutritional roles. Terpenoids "as group of secondary metabolites" are engaged in several physiological and ecological processes and are one of the biggest diversified groups in the kingdom of plants. This study provides an overview of terpenoid metabolism and highlights current developments in the scope of terpenoid metabolic engineering. Plants often only produce terpenes in certain tissues or cell types, such floral organs. There is a lot of potential in using various plant metabolic engineering techniques to control the terpenoids' production. There are several ways to increase the availability of rare terpenoids, lower the cost of pricey medications, and raise people's standards of life.

KEYWORDS: Metabolic Engineering; Secondary Metabolites; Terpenoids Metabolism, Future Perspectives; Metabolic Engineering Strategies.

1. INTRODUCTION

1.1. Evolution Of Metabolic Engineering

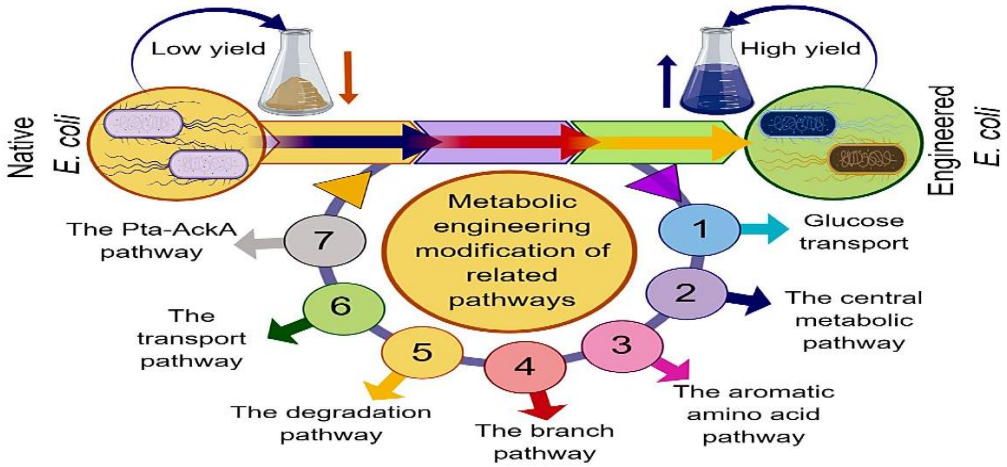

Fig. 1: Schematic Representation of Metabolic Engineering Approaches for Induced Production Of L-Tryptophan Using Engineered E. Coli According to Liu Et Al., 2019.

Globally speaking, metabolic engineering is like cellular and genetic engineering in that their shared goal is to modify genes to produce a desired result. The deliberate creation, rerouting, and alteration of cellular metabolism to provide desired molecules is known as metabolic engineering (**Fig. 1**) (**Liu et al., 2019**). All a cell's biochemical operations are referred to as cellular metabolism, and they are made up of metabolic pathways, which are collections of enzymatic steps. Genetic and cell engineering, on the other hand, concentrate on the specific enzymatic processes occurring within the cell, while metabolic engineering is primarily concerned with carefully comprehending the broader metabolic network. Because of their organizational structures, prokaryotes and cultured eukaryotes are easier to work with than multicellular species. This is because metabolic engineering is focused on altering the metabolic pathways of unicellular organisms. The ability of a production organism to effectively produce the desired product is the primary factor considered when choosing one for an industrial biotechnology process (**Chroumpi et al., 2025)**

The foundation of metabolic engineering is altering the metabolic pathways of unicellular organisms, especially prokaryotes and cultured eukaryotes, which are simpler to work with than multicellular species due to their organizational structures. Numerous metabolic processes found in plants oversee producing complex compounds through biosynthesis **(Zhu et al., 2021)**. To produce nonprotein metabolites, prokaryotes, and cultures of simple eukaryotes, such as yeast, filamentous fungus, and plants, are more commonly used than complex eukaryotic cultures. Comparing the genomes and organelles of the two groups reveals the intracellular characteristics that set them apart. While eukaryotes, such as the kingdoms of fungi, plants, and animals, have multiple DNA molecules arranged as closely bound chromosomes and have organelles surrounded with membranes (e.g., the Golgi apparatus, chloroplasts, and mitochondria), prokaryotes, which include the kingdom of bacteria, are devoid of these organelles and contain a single intrinsic single loop of stable chromosomal DNA. In metabolic engineering applications, plants, animals, and bacterial cells are all utilized to create commodities **(Guo et al., 2025).**

Prominent industrial metabolic engineering products include biofuels, biodegradable and biocompatible polymers, recombinant proteins, and amino acids. A series of biopolyesters with adjustable strengths, biodegradability, biocompatibility, and thermoprocessibilty are called polyhydroxy alkanoates, or PHAs. They are produced by bacteria. PHAs are used in medical implants, medication delivery vehicles, coatings, films, boxes, and fiber and foam materials, among other packaging materials. Recently, recombinant PHA-producing strains with improved mechanical and thermal properties have been created by applying strategies including metabolic engineering and process improvement (**Paduvari & Somashekara, 2025).**

Organic compounds known as secondary metabolites are those that do not directly participate in the essential metabolic pathways that control regular cell division, growth, and reproduction. For many years, the industry has effectively boosted the output of desired products using traditional strain enhancement procedures. For instance, the yeast organism Penicillium chrysogenum, which makes penicillin, has been altered to increase output by

around 1000 times. To significantly boost the productivity of industrial strains, rational metabolic engineering design has been increasingly important in recent years. Strategies incorporate ribosome engineering, precursor modification, downregulation, overexpressing organizational genes, upregulation, boosting self-resistance, and articulating biosynthetic gene groups. By employing these strategies, secondary metabolite production improves significantly. For instance, overexpression of actinorhodin has caused a 470-fold rise in ***Streptomyces lividans*** production **(Pham et al., 2021)**.

The manufacturing of secondary metabolite medications, such as those with antiviral, antioxidant, anticancer, and antitumor properties, also employs metabolic engineering techniques (**Habeballa, et al., 2020, Alhaithloul et al., 2022 & 2023)**. Furthermore, cubebol, valencene, torulene, patchoulol, astaxanthin, carvone, artemisinic acid, lycopene (an antecedent of artemisinin, an antimalarial medication), echinomycin, ivermectin, parathyroil, growth hormone in humans, and indolocarbazole compounds have all been synthesized with the help of metabolic engineering techniques **(Seca & Pinto 2018)**. Shikimic acid and quinic acid are essential chiral starting components required to synthesize a neuraminidase inhibitor to treat influenza. These acids are often derived from plants **(Wu et al., 2022)**.

The most varied class of natural compounds, terpenoids, are the subject of our attention here. More than 40,000 distinct terpenoids have been identified **(Roba 2020).** These substances, which include sterols, the hormones abscisic acid, chlorophylls, carotenoids, brassinosteroids, and gibberellins, are necessary for fundamental functions including development, and photosynthesis as well as growth. Furthermore, crucial intermediaries of interactions between plants and their biotic surroundings are terpenes. They are sometimes referred to as di-, sesqui-, and mono- terpenes (C20, C15, and C10, respectively). Since many of these terpenes are volatile, they appeal to seed dispersers and pollinators as well as contribute to defense of plants against infections and herbivores both directly and indirectly (**Li et al., 2024**).

1.2. Biological Systems

The ability of a production organism to effectively produce the desired product is the primary factor considered when choosing one for an industrial biotechnology process. Two main categories of products are produced by genetically modified cells: nonproteins and proteins (**Balabanova et al., 2015**). Encoding genes for proteins are inserted into plasmids or the host genome to produce products of proteins. These produces can be employed as industrial catalysts, human treatments, animal husbandry, or food processing. To produce nonprotein yields, fundamental enzymes that convert precursors into desirable metabolites—such as vitamins, biofuels, amino acids, and antioxidants—must be introduced by the introduction of genes. Plants and yeast (as a filamentous fungi) are examples of prokaryotes and cultures of basic eukaryotes that are more commonly employed to manufacture nonprotein compounds than complex eukaryotic cultures that are used to produce proteins.

To boost output, one must be able to alter an organism's genetic makeup (plasmid DNA or endogenous genome), the machinery of the cell must be able to manage the result, and the final product must be recoverable and purifiable. Well-characterized physiology and genome make microorganisms particularly desirable since it is easier to alter them to increase productivity. An excellent starting point for complex genetic modification is the availability of a large variety of hosts with characterized genomes, comprising promoters, transcriptional regulators, and combinations of mutations. When choosing a host, it is also better to know how the organism responds to physiological changes, such as changes in substrate and process conditions. In the process of synthesizing a desired product, each host has advantages and drawbacks built on its physiology, metabolism, and endogenous genome. Thus, when the pathway of interest's features shows a main position in the selection procedure, economically efficient manufacturing is attained **(Abualreish & Abdein, 2014 and Kim et al., 2024).**

Advantages And Disadvantages of Perfect Host

Since the selection of expression system and host organism impacts how effectively targeted production will continue, every cellular component that affects the process needs to be considered. These include understanding the genetic code, secretion ability, growth rate, degree of product expression, cost of cell growth (including growth media, supplements, growth vessels, and special handling expenses), and ability to fold and modify proteins appropriately (**Fakruddin et al., 2013).** It is easier to control microorganisms that have undergone considerable research and characterization to maximize output. Sequenced genomes, pre-existing

mutant libraries, proteome maps, well-characterized physiology and biochemistry, and well-established genetic methods make these model hosts valuable. The quality and speed of genetic changes are enhanced, manufacturing and posttranslational modification capabilities are advanced, and this information is easily accessible.

1.3. A Bioreactor

Any manmade system or instrument that facilitates a biologically active environment is called a bioreactor. In one instance, a bioreactor is a container used to conduct a chemical reaction involving living things or materials that are biochemically active and produced from living things. Anaerobic or aerobic conditions might apply to this procedure. In cell culture, the term "bioreactor" can as well as denote to a tool or technique intended to produce tissues or cells. These bioreactors are usually made of stainless steel and have a cylindrical form. Their sizes range from cubic meters to liters. Tissue engineering and biochemical/bioprocess engineering will be able to employ these new instruments. Bioreactor types include batch, fed batch, and continuous (such as continuous stirred-tank reactors) based on how they operate. A particular kind of continuous bioreactor is the chemostat (**Kowalczyk et al., 2021**).

In bioreactors, organisms or biochemically active materials can be fastened to the outside of a solid media or immersed in a liquid medium. Cultures submerged in water might become suspended or immobile. Because they don't require specific attachment surfaces, suspension bioreactors have the potential to accommodate a greater range of species and can function on a far bigger scale than immobilized cultures. However, a continually functioning approach will be used to remove the organisms from the reactor along with the runoff. "Immobilization" encompasses a wide range of methods for binding or ensnaring cells or particles. Almost all forms of biocatalysis, such as those involving enzymes, cellular organelles, and the cells and organs of plants and animals, may use it. Because the bacteria are restricted to the vessel's surfaces, immobilization is beneficial for procedures that are run continuously because the organisms won't be eliminated with the reactor effluent (**Fig. 2 a & b**) **(Decker & Reski 2007; Baranwal et al., 2022)**. The following are examples of large-scale arrested cell bioreactors: packed bed, membrane, packed bed biofilm reactor (MBBR), and moving media.

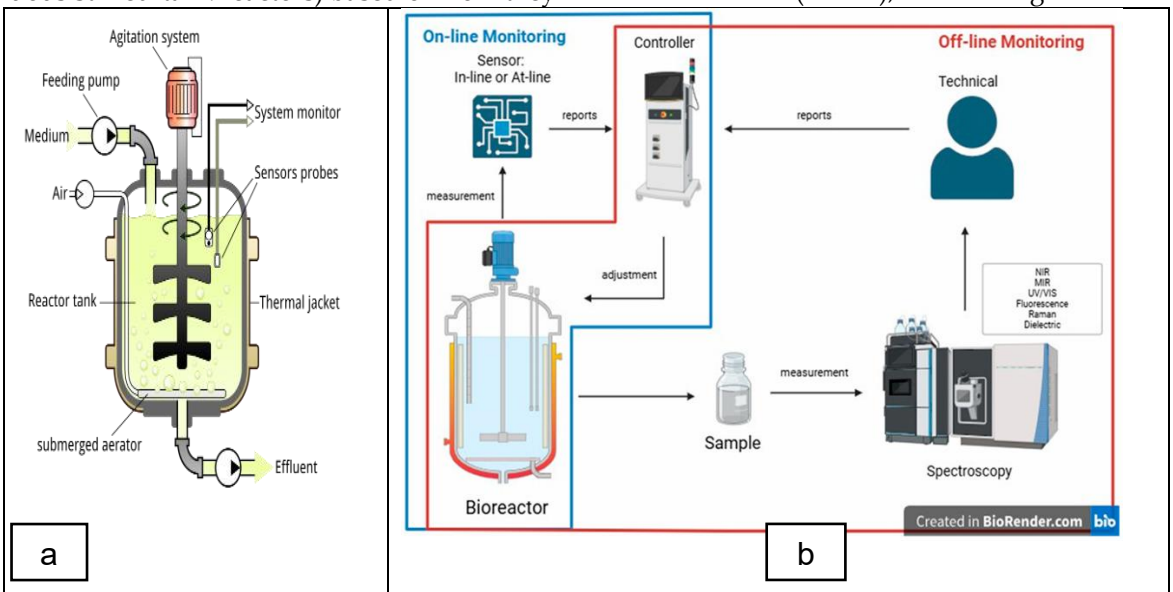

Fig. 2, A: A Continuous Stirred-Tank Type Bioreactor's General Construction (Decker & Reski 2007).
B: Schematic Of an On-Line and Off-Line Monitoring System (Baranwal Et Al., 2022).

2. USING ENGINEERED YEAST TO PRODUCE PHARMACEUTICAL TERPENOIDS VIA FERMENTATION TECHNIQUES.

Saccharomyces cerevisiae is a viable cell factory due to developments in biochemistry, industrial terpenoid biosynthesis, and omics research (genomics, transcriptomics, metabolomics, proteomics). Furthermore, the development of fermentation is a major factor in the high titer, yield,

and productivity (TYP) of these compounds. Recent research on the recombinant synthesis of medicinally significant terpenoids by modified Saccharomyces *cerevisiae*, was compiled by **Carsanba et al. (2021**), with an emphasis on fermentation techniques to boost TYP to satisfy industrial needs and supply the pharmacological market. Reviewing strain design and fermentation settings, as well as discussing batch, fed batch, and continuous fermentation processes, are factors that impact the generation of recombinant terpenoids.

3. BIOSYNTHESIS PATHWAY OF TERPENOIDS IN PLANTS

The synthesis of terpenes in plants is frequently limited to certain tissues or cell types, such as glandular trichomes, which are hair-like projections on the surface of leaves, stems, and fruits, or floral organs. Plant-derived terpenoids, including ginkgolides, taxol, and artemisinin, are effective healers of a range of illnesses. The most successful treatment for drug-resistant strains of *Plasmodium falciparum*, the parasite that causes cerebral malaria, is now artemisinin, a sesquiterpenoids lactone produced from *Artemisia annua* **(Pan et al., 2018)**. A well-known anticancer drug, paclitaxel, sometimes referred to as taxol, is a diterpenoid made by *Taxus brevifolia* and other *Taxus* species **(Lenka et al., 2012)**. Platelet-activating factor receptor antagonists with high specificity belong to the physically distinct class of diterpenoids known as ginkgolides. However, because of their poor yield from natural sources, pharmaceutical terpenoids' commercialization is currently restricted**.** Many therapeutic plants have sluggish growth rates and are sensitive to their surroundings. Certain therapeutic plants are threatened with extinction and cannot be collected to separate their terpenoids for use in pharmaceuticals. Moreover, there are often significant changes in pharmaceutical terpenoids' levels, which makes medication extraction and quality monitoring difficult. The whole chemical production of these intricate molecules is expensive, though (**Zhu et al., 2021; Cheng et al., 2025).**

Isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), are the source of all terpenoids and are produced by plants via two distinct processes **(Hemmerlin et al. 2012).** The methylerythritol-phosphate (MEP) pathway is used by plasmids, while the mevalonic acid (MVA) system is partially present in peroxisomes and the cytoplasm. Once larger prenyl diphosphate intermediates are produced by prenyl transferases using IPP and DMAPP, terpene synthases (TPSs) employ these substrates to make mono-, sesqui-, and diterpenes. Different prenyl diphosphate isomers may arise when one DMAPP molecule condenses head-to-tail with one or more IPP molecules. If the isoprene units are connected in a trans (E) or cis (Z) arrangement, this might occur. Long known to be connected in the cis (Z) conformation are the isoprene units of various long-chain plant terpenoids, including rubber and dolichols, as they are derived from Z-prenyl diphosphates generated by cis-prenyl transferases **(Akhtar et al., 2013)**. But geranyl diphosphate (GPP), which is created when one DMAPP and one IPP molecule are joined in the trans (E) configuration, is well recognized to be the substrate for monoterpene biosynthesis by TPSs **Fig. (3)(Pazouki & Niinemets, 2016)**. It has long been thought that all-trans-farnesyl diphosphate (E, E-FPP) was the only precursor that sesquiterpene synthases could use to produce sesquiterpenes **(Couillaud et al., 2023)**.

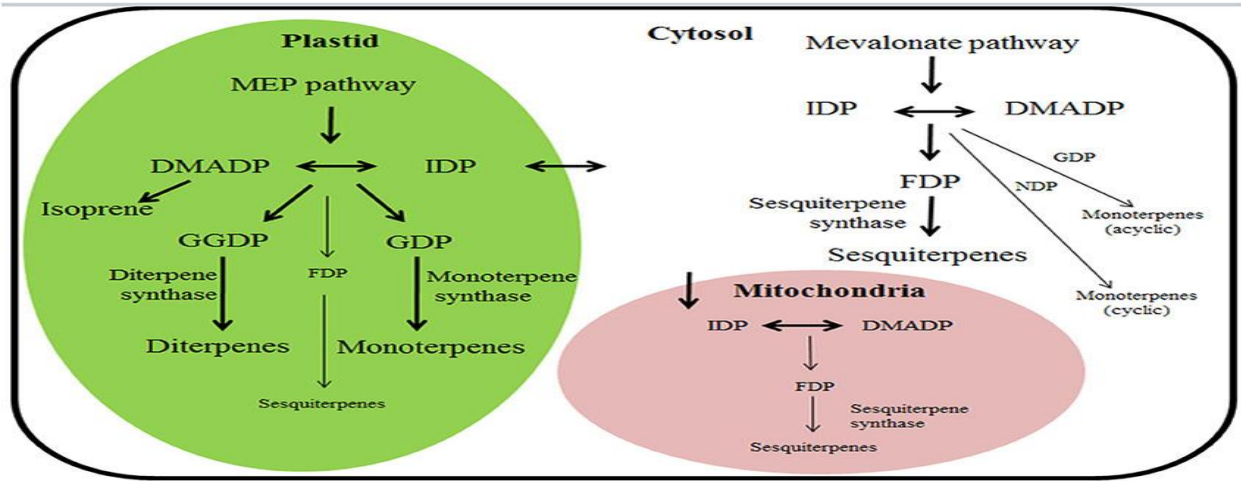

Fig. 3: Terpene Biosynthetic Pathways and Their Subcellular Compartmentalization in Plants. Thick Arrows Denote The Classical Understanding Of Terpenoid Synthesis Compartmentalization Among Cytosol And Pla stid According To (Pazouki & Niinemets, 2016).

4. TECHNIQUES FOR PLANT METABOLIC ENGINEERING

The three main goals of metabolic engineering in plants are to create a novel compound (a molecule produced in nature but not typically found in the host plant), increase the production of a desired compound, and decrease the production of an unwanted compound **(Mipeshwaree et al., 2023)**. Creating shortcuts that redirect metabolic flux in a certain direction, blocking alternative pathways, or engineering phases in a pathway to adjust the metabolic flow to target molecules are some techniques to achieve these goals. This strategy, however, is only partially effective since the system frequently absorbs the consequences of changing individual enzymatic processes to return equilibrium. Controlling metabolic flow in a more predictable way may be possible by focusing on different phases in the same pathway. This might involve upregulating many enzymes in a route sequentially, suppressing those in a competing pathway while upregulating those in the first, or using regulatory genes to achieve multipoint control over one or more cell processes (**Fig 4**) **(Lu et al. 2016).**

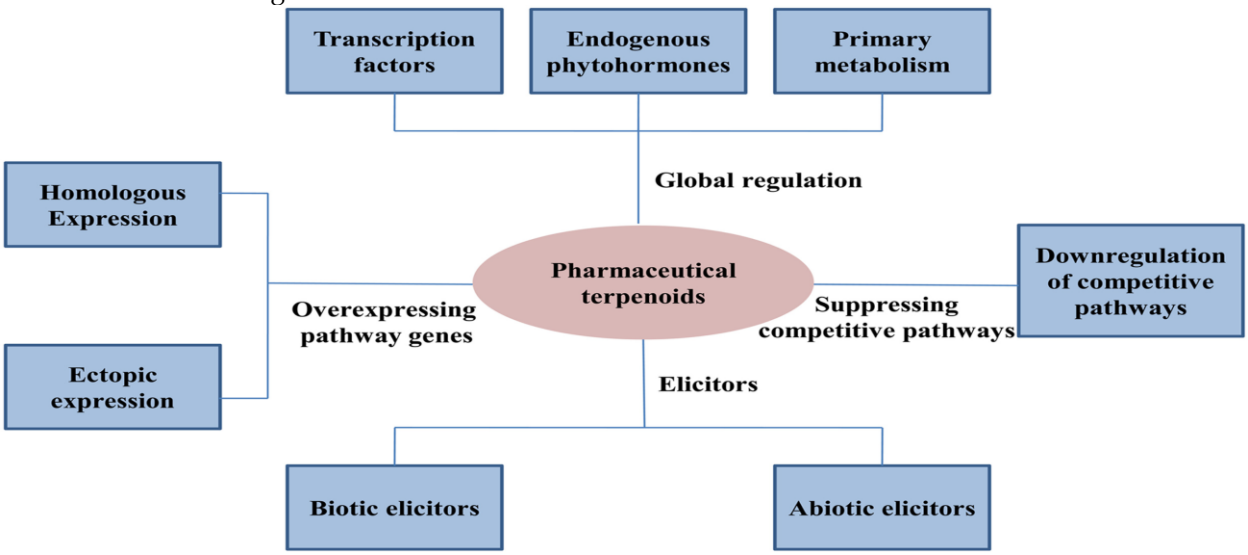

Fig. 4: Techniques For Plant Metabolic Engineering to Control Medicinal Terpenoids Including (1. Global Regulation, 2. Overexpressing Pathway, 3. Suppressing Competitive Pathways And 4. Elicitors). According To Lu Et Al. 2016.

4.1. An Elicitor

A chemical, either endogenous or exogenous, stimulates higher plants to produce phytoalexins. Throughout development, plants are subject to strict regulatory control on the manufacture of secondary metabolites. These metabolites accumulate more while under stress or when microbes assault **(Isah, 2019)**. Elicitation is a highly alluring method for raising the productivity of metabolites in in vitro growth systems. Shorter production periods and higher yields can result from eliciting plant cells or tissue **(Hashim et al., 2021**). Secondary metabolism links the synthesis of metabolites with the plant defense system **(Isah, 2019)**. Therefore, the activation of defense mechanisms produced by biotic elicitors may result in an increase in the accumulation of secondary metabolites.

Terpenoids found in pharmaceuticals derived from medicinal plants are frequently endogenous defense metabolites. To enhance the generation of desired terpenoids and promote secondary metabolism, biotic and abiotic elicitors can be employed.

4.1.1. Application Of Biotic Elicitors in Cell Culture:

Biotic elicitors are derived from live creatures, including fungus, bacteria, and viruses. Involved in the reaction to elicitors, hormones of plants (e.g., salicylic acid, abscisic acid, and jasmonates) can be utilized in place of biotic elicitors (**Cusido et al., 2014**, **Shoala, et al., 2021**). The synthesis of terpenoids is significantly aided by several biotic elicitors. According to **Lin et al. (2023)**, *G. biloba* cell suspension cultures' secondary metabolite accumulations were successfully elevated by a natural bacterial elicitor. Ginkgolide B, ginkgolide A, and bilobalide levels rose 1.5, 2.6, and 2.1 times, relative to the control group, after a 24-hour intervention with naturally occurring Staphylococcus aureus. A-naphthalene acetic acid-supplemented Murashige and Skoog (MS) medium

was used by **Abramov (2019)** to establish an adventitious root development strategy utilizing Andrographis paniculata leaf segments. Compared to a normal cell suspension culture, the amount of rographolide (diterpenoid) was 3.5 times greater. When the adventitious root culture of *P. campestris* was grown for seven days, the highest concentration of triterpenoid (maytenin) was 972 mg/g DW (**Paz et al., 2013**). Another crucial component in the production of the significant anti-cancer medication paclitaxel is biotic elicitors. The endophytic fungus *Fusarium mairei*, which grows on *Taxus chinensis* suspension cells, was co-cultured with them to manufacture paclitaxel in a 20-liter bioreactor. Compared to uncoupled culture, the output is 38 times higher (**Cao et al., 2022**)**.** To increase paclitaxel production in cell culture, several biotic elicitors are used. MeJA (methyl jasmonate) or coronatine can efficiently boost the synthesis of paclitaxel in *Taxus* cell suspension cultures, even if doing so may inhibit the development of the suspension cells **(Xie et al., 2025).**

4.1.2. Abiotic Elicitors

They consist of metal ions and inorganic substances like copper sulphate and silver nitrate, among others. One diterpenoid lactone with significant pharmacological properties is andrographolide, which has anti-inflammatory, anticancer, and anti-HIV properties (**Tundis et al., 2023**). According to **Gandi et al. (2012),** andrographolide concentration in *A. paniculata* suspension cultures was increased by using abiotic elicitors ($AgNO_3$, $CdCl_2$, $HgCl_2$, and $CuCl_2$). Of all those metal salts, andrographolide production was most affected by the $CdCl_2$ treatment.

In suspension cultures of P. *ginseng*, the synthesis of triterpenoid ginsenosides was effectively increased by the application of abiotic elicitors. **Huang and Zhong (2013**) claim that salts of heavy metal such ammonium metavanadate, vanadyl sulfate, $MnSO_4$, and $NiSO_4$, stimulated the manufacture of ginsenosides. Out of all the therapies studied, vanadate was shown to be the most effective. Further analysis showed that vanadate supplementation increased the transcription levels and endogenous JA production of the genes encoding dammarenediol-II synthase (DS) and squalene epoxidase (SE).

4.2. Overexpression Of Terpenoid Biosynthesis Key Genes:

4.2.1. Homologous Overexpression

Overexpressing Pathway of Single Gene:

Since the terpenoid compound production is strictly regulated by the enzymes involved in the biosynthesis pathways, regulating the expression of these genes might result in higher productivity **(De Geyter et al., 2012)**. The primary pharmacologically active components of *Panax ginseng* are ginsenosides, a class of triterpenoids that are further classified as protopanaxadiols and protopanaxatriols **(Ratan et al., 2020)**. Recently, many genes implicated in the route of ginsenoside synthesis in Panax ginseng have been cloned and overexpressed. A twofold increase in phytosterols and a 1.6–3 times increase in total ginsenosides were observed in transgenic ginseng adventitious root cultures because of overexpressing the critical gene PgSQS1, which is involved in the pathway involved in the biosynthesis of ginsenosides and can up-regulate the expression of SE, b-amyrin synthase (b-AS), and cyclo-artenolsynthase (CAS). However, compared to non-transgenic adventitious roots, transgenic ginseng roots grow more slowly **(Kowalczyk et al., 2022**). The amount of oleanane-type ginsenoside (ginsenoside Ro) in *Panax ginseng* was shown by **Han et al. (2013)** to be greatly increased by overexpressing the ginsenoside biosynthetic key gene CYP716A52v2, although other dammarene-type ginsenoside levels were like control lines.

Increasing research combines these metabolic control techniques to generate objective terpenoids. For example, *Taxus* X medium hairy root cultures with limited growth potential were created by overexpressing the *Taxus baccata* TXS gene. To address this issue, hormone therapy was used to dedifferentiate transgenic hairy roots into callus. Cell suspension lines were also developed. According to **He et al., 2023** HR formation of *T. baccata* was successfully achieved by natural transformation using the *R. rhizogenes* A4 strain, with a transformation efficiency of 14.3% generating one HR. This breakthrough finding revealed for the first time that the *R. rhizogenes* A4 strain is in fact capable of transforming Taxus spp., which paves the way for establishing a platform for producing paclitaxel. Additionally, it also closes the knowledge gap that *T. baccata* is susceptible to transformation with R. rhizogenes.

4.3. Cooperated Expression of Several Genes

Pharmacological terpenoids in medicinal plants may be efficiently increased by metabolic control of many important genes in biosynthesis pathways. In *Salvia miltiorrhiza*, tanshinone are norditerpenoid quinines of the abietane class. They possess extensive

anticancer activity, anti-inflammatory, and antimicrobial properties (**Gao et al., 2014**). Tanshinone production is significantly increased in *Salvia miltiorrhiza* hairy root lines upon insertion of the SmHMGR and/or SmGGDS gene in conjunction with the SmDXS gene **(Wei et al., 2019)**. Tanshinone production was greatest when the SmGGDS and SmHMGR genes were co-expressed, almost 4.7 times greater than the control. The artemisinin concentration (in *Artemisia annua*) was 2.4 times greater than that of the untreated plants (**Lu et al., 2016**).

4.4. Expression Of Biosynthetic Genes Ectopically.

It can be challenging to genetically modify some therapeutic plants that yield little amounts of pharmaceutical terpenoids in their natural form. To create therapeutic terpenoids in various plants, ectopic expression of terpenoid synthases and cyclases (TPSs) has been utilized by certain researchers. Due to its pleasant rose-like scent, geraniol monoterpenoid alcohol has significant economic value in the fragrance industry. It may also be utilized as an antibacterial reagent and an anticancer medication **(Fajdek-Bieda et al., 2024).** By using the *Valeriana officinalis* geraniol synthase gene (VoGES) in tobacco hairy root cultures, geraniol may be produced. According to metabolic studies, geraniol derivatives were found in six main glycoside types. Total geraniol levels after deglycosylation were 204 mg/g DW **(Ritala et al. 2014)**. Spike lavender oils (*Lavendula latifolia*) with a high linalool concentration and low camphor level are perfect for the fragrance and cosmetic sectors. The transgenic *Lavandula latifolia* young leaves exhibited a considerable rise in linalool concentration, with an increase of up to 10-fold **(Mendoza-Poudereux et al., 2017).** The concentrations of (E)-a-bergamotene in transgenic *Nicotiana attenuata* were 2- to 25-fold higher than those in non-treated plants.

Hasan et al. (2014) discovered that the TbTS gene was constitutively overexpressed in *Nicotiana benthamiana*. Triterpenoids may produce more when other terpenoid biosynthesis genes are overexpressed ectopically. Overexpression of Panax ginseng HMGR1 caused 1.5–2.5 times higher platycoside yields and 1.1–1.6 times higher phytosterols in *Platycodon gradiflorum* hairy by ectopic transformation mediated by *Agrobacterium rhizogenes* (**Kim et al., 2014)**. Some investigators overexpressed PgDS, or PgDS + PgCYP716A47, an important gene involved in ginsenoside production, in transgenic tobacco plants. Transgenic tobacco roots collected more dammarenediol-II than leafy stems, flower buds, or stems because the PgDS gene was overexpressed. The transgenic line's root may produce up to 158 mg/g DW of dammarenediol-II. Transgenic tobacco leaves exhibited quantities of protopanaxadiol (PPD) ranging from 2.3 to 5.7 mg/g DW when PgCYP716A47 and PgDS, the CYP450 gene implicated in the ginsenosides biosynthesis pathway, were co-overexpressed **(Chun et al., 2015)**.

4.5. Suppressing Competitive Pathways

4.5.1. The Inhibition of Competitive Pathways to Increase Terpenoids' Manufacturing.

Suppressing the expression of competing metabolic pathways is another tactic. In *Artemisia annua*, the sterol route competes with artemisin' s production. Because RNA interference (RNAi) decreased the expression of SQS, a critical gene in the sterol pathway, transgenic plants exhibited much greater levels of artemisin. The maximum amounts of artemisin were almost 3.14 times more than those of the control plants (**Ali et al., 2017**). Three genes are implicated in the competitive branch pathway: germacrene, b-caryophyllene synthase (CPS), and b-farnesene synthase (BFS). The antisense method further downregulated *Artemisia annua* GAS, the synthase gene, and SQS independently. In several transgenic lines, there were notable increases in the amounts of artemisin and dihydroartemisinic acid (DHAA). Levels of DHAA and artemisinin rose by 132% and 77%, respectively, in anti-CPS transgenic plants. Transgenic plants against BFS exhibited 54% and 77% increases in DHAA and artemisinin levels, individually **(Zhao et al., 2022**). For human health, carotenoids, also known as tetraterpenes, are essential because they perform a diversity of tasks in the planta kingdom. The first gene in the competitive branch route, lycopene +-cyclase (LCY-+), was downregulated by RNA interference (RNAi) to enhance carotenoid production via the b-branch-specific pathway **(Kim et al., 2014).** It was demonstrated that whereas the lutein concentration in the transgenic calli was decreased to undetectable levels, the b-carotene content in the sweet potato transgenic calli was around 21 times greater than in the control (**Xia et al., 2022**)**.**

5. GLOBAL REGULATION

5.1. Controlling Transcription Factor Expression

Kajla et al. (2023) state that transcription factors may influence the quantity of secondary metabolites that plants generate in addition to playing significant

roles in the genetic transcription related to biosynthesis. It has previously been demonstrated that several transcription factors have global regulatory roles in terpenoids.

5.1.1. Ap2/Erf Transcription Factors

Numerous plant species' metabolisms are regulated by these transcription factors, which also affect how plants respond to biotic and abiotic stimuli. They feature a preserved connecting domain consisting of 57–66 amino acids, according to **Kajla et al. (2023)**. These transcription factors have been the subject of recent artemisinin metabolic engineering studies. The RAV1AAT(RAA) and CRTDREHVC BF2 (CBF2) motifs of the ADS and CYP71AV1 promoters are binding sites for AaERF1 and AaERF2. The amounts of artemisinic acid and artemisinin were increased by their overexpression in transgenic *Artemisia annua* **(Yu et al., 2012)**. There was a notable rise in DHAA and artemisinin when the trichome-specificAP2/ERF transcription factor AaORA was overexpressed. Additionally, these transgenic *Artemisia annua* showed increased resistance to the phytopathogenic fungus *Botrytis cinerea* (**Lu et al., 2013)**. Trichome and Artemisinin Regulator 1 (TAR1), another AP2/ERF transcription factor, was cloned from *A. annua* and binds to the cis-acting regions of the CYP71AV1 and ADS promoters. **Tan et al. (2015)** reported that transgenic *A. annua* lines expressing overexpression of TAR1 exhibited a noteworthy increase in artemisinin levels.

5.1.2. Transcription Factors of WRKY

The WRKY family of transcription factors, which specifically bind to the W-box of promoters, governs developmental processes in plants, including senescence, metabolism, in addition to defense responses. *Artemisia annua*'s GST cDNA library was used to clone AaWRKY1 **(Zhang et al., 2021)**. Compared to wild-type plants, trichome-specific AaWRKY1 overexpression resulted in up to 33 times more CYP71AV1 transcription. However, the substantially up-regulated CYP71AV1 in transgenic plants improved the synthesis of artemisinin by almost 1.8 times **(Behera et al., 2023).**

5.1.3. Factors Involved in Transcription for Basic Helix-Loop-Helix (Bhlh)

Basic helix–loop–helix (bHLH) transcription factors are found in all eukaryotic species and are involved in several regulatory processes. Iridoids are a class of monoterpenoids found in iridoid sand that include secologanin and loganic acid. Numerous of these substances possess bioactive properties of their own, including anti-inflammatory, antibacterial, and anticancer properties (**Viljoen et al., 2012)**. Cloning *C. roseus* allowed for the creation of the jasmonate-regulated bHLH transcription factor. Genes that produce the iridoids loganic acid and secologanin, which are produced from the terpenoid precursor GDP, may be transactivated by BIS1. In *C. roseus* suspension cell cultures, BIS1 overexpression increased the production of high value iridoids and monoterpenoid indole alkaloids (**Van Moerkercke et al., 2015**). BIS1, BIS2, and other bHLH TFs that respond to jasmonate (JA) can be coupled to form heterodimers or homodimers that transactivate the promoters of genes that are involved in the synthesis of iridoids. Cloned from a GSTcDNA library, AabHLH1, the AbHLH transcription factor from *A. annua*. The yeast transactivation protein AabHLH1 binds to the E-box cis-elements in the promoters of both ADS and CYP71AV1. ADS, CYP71AV1, and HMGR transcript levels were elevated in *A. annua* leaves with transient expression of AabHLH1, all of which are implicated in the artemisinin manufacture **(Ji et al., 2014)**. Recent research shown that AaMYC2 was rapidly activated by JA and could bind to G-box-like motifs found in the promoters of DBR2 and CYP71AV1. In transgenic ***A. annua***, overexpression of AaMYC2 markedly enhanced the amount of DHAA and artemisinin relative to the WT.

5.2. Controlling The Endogenous Phytohormone Levels Associated with Terpenoid Biosynthesis

Often, pathogen invasion or herbivore grazing stimulates terpenoid production **(Purnomo, 2021)**. Jasmonate hormones (JAs) are essential components of a complicated signaling cascade that regulates the transcriptional response **(Li et al., 2022)**. Consequently, it may be possible to regulate the synthesis of therapeutic terpenoids in plants by using the jasmonate biosynthetic pathway. The main enzyme in the process that produces jasmonate, allele oxide cyclase (SmAOC), was overexpressed in *S. miltiorrhiza* hairy root cultures. Furthermore, jasmonates are necessary to regulate the synthesis of artemisinin. Elevated endogenous JA led to a significant rise in the synthesis of artemisinin, DHAA, and artemisinic acid **(Lu et al., 2014**). Nevertheless, this strategy's use is restricted since high JA concentrations in plants may impede their growth. Abscisic acid (ABA), a phytohormone crucial to plant growth and response to environmental stress, may influence the synthesis of medicinal terpenoid compounds. The tomato SlNCED1 gene is inhibited by an RNA interference construct that has an E8 promoter unique to the fruit. When compared

to the control, the transgenic tomato's SlNCED1 mRNA levels were down-regulated by 20–50%, partially obstructing the carbon flow that leads to free ABA. The reduce in endogenous ABA led to a raise in ethylene production because more genes implicated in the ethylene biosynthesis pathway were transcriptionally activated throughout the ripening stage. Increased buildup of lycopene and beta-carotene was the outcome of the inhibited carbon flow's impact on the RNAi lines' carotenoid pathway. As a result, during ripening, tomato fruit from the RNAi lines had a deeper red hue than the untreated fruit (**Sun et al., 2012**). According to **Lu et al. (2011**), ABA therapy may also boost the synthesis of artemisinin. The full-length cDNA of *A. annua,* which codes for the ABA receptor AaPYL9, was cloned and examined. **Zhang et al. (2013**) report that following ABA treatment, overexpression of AaPYL9 boosts the production of artemisinin and enhances drought tolerance. There is a noticeable rise in the expression of genes related to artemisinin production.

5.3. Adjusting Linked Primary Metabolism

Controlling the pertinent primary metabolism of medicinal plants can also increase the output of terpenoids. Glucose is one of the main products of basic metabolism. A thorough statistical study revealed that carbohydrates had a significant effect on the production of geraniol in transgenic tobacco cell suspension cultures. When sucrose was used instead of glucose and D-mannitol, the cell cultures generated the highest quantity of geraniol and biomass accumulation. **Biswas et al., (2023)** found that geraniol production was greatly impacted by light, which facilitates the process of photosynthesis, which creates carbohydrates. Overexpression of neutral/alkaline invertase gene, a crucial gene for the hydrolysis of sucrose, has been shown to significantly upregulate the expression of the Taxa diene synthase gene (TAS) in *Tsuga chinensis* cells.

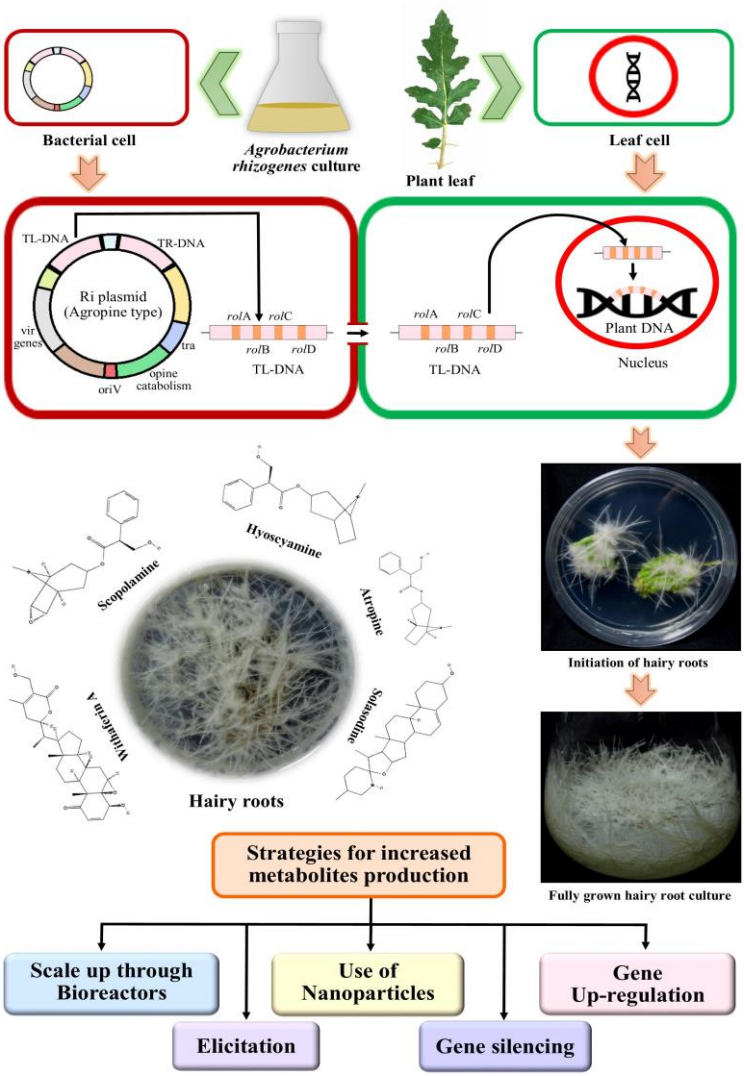

Fig. 5: Strategies For Increased Metabolites Production According to Biswas Et Al., 2023.

6. CONSTRUCTING NOVEL METABOLIC ROUTES

The pathways already present in the host plant are often altered or expanded by metabolic engineering, but multigene engineering may also be utilized to introduce whole new pathways and produce entirely alien products. One study used sorghum (*Sorghum bicolor*) to manufacture uridine diphosphate glucose (UDPG)-glucosyltransferase and two multifunctional cytochrome P450 enzymes. The cyanogenic glucoside dhurrin, which confers resistance to the flea beetle (*Phyllotreta nemorum*), was subsequently produced by injecting these enzymes into *Arabidopsis thaliana* **(Zagrobelny et al., 2018)**. In addition, the researchers created transgenic plants that did not have UDPG-glucosyl transferase but did have one or both cytochrome P450 enzymes. These plants showed the accumulation of the proper metabolic intermediates.

These groundbreaking attempts to engineer terpenoids from plants showed that it is feasible and even beneficial to produce plant terpenoids in algae **(George et al., 2020)**. However, they also brought attention to the necessity of critical bottlenecks that provide sufficient substrate pools and metabolic pull from heterologous production with competing vital pathways, such as sterol biosynthesis. The need for efficient engineering to understand and characterize endogenous isoprenoid metabolism is highlighted by this. Because they can transmit massive extrachromosomal genetic constructs, diatoms are among the most promising alternatives among photosynthetic eukaryotic microorganisms. For heterologous synthesis in these species, this opens more complex and multiplexed engineering strategies (**Vavitsas et al., 2018**).

7. ENGINEERING TERPENOIDS PATHWAYS

Terpenoids, a family of structurally diverse polymers generated from isoprene, are sometimes referred to as isoprenoids. Depending on how many isoprene units they contain—which rises by fives—they are grouped. As a result, monoterpenes contain ten isoprene units, sesquiterpenes have fifteen, diterpenes have twenty, and hemiterpenes have five. Terpenoids containing forty isoprene units are called carotenoids. The biggest polymers, which include natural goods like rubber, include thousands of units. Despite their differences, isopentyl pyrophosphate and dimethylallyl pyrophosphate are the same precursors that are used to make all terpenoids. Despite their differences, dimethylallyl pyrophosphate and isopentyl pyrophosphate are the same precursors used to make all terpenoids. Metabolic engineering has focused on the mevalonate and the mevalonate-independent pathways, which are the two different mechanisms by which this happens (**Lu et al., 2021**).

Though see Update, there are currently few examples of monoterpenoid metabolic engineering. One project involved inserting the *Clarkia breweri* (S)-limonene synthase gene into petunia plants genetically to cause them to generate the insect repellant linalool **(Fig. 5)**. In another, a fruit-specific promoter controlled the introduction of the same gene into tomatoes, which led to the buildup of linalool in tomato fruits **(Boncan et al., 2020).**

The carotenoid metabolic pathway has received a great deal more attention. Pigments produced by this mechanism are vital for animal and human nutrition and are involved in photoprotection and light harvesting (**Swapnil et al., 2021**). The starting point for the synthesis of carotenoid pigments in plants is geranylgeranyl pyrophosphate (GGPP), which is produced when two geranyl phosphate molecules join. The enzymatic process transforms this into phytoene, which is then converted into δ by phytoene desaturase beta-carotene. Lycopene is made by additional desaturation, which is subsequently transformed into β-carotene (provitamin A) by the enzyme β-cyclase, or it can be made via a sequence of enzymatic reactions that are catalyzed by β-cyclase and ε-cyclase into α-carotene and δ-carotene **(Rosas-Saavedra et al., 2023).**

The synthesis of β-carotene in the endosperm of rice (Golden Rice) is undoubtedly the most notable example of carotenoid metabolic engineering in plants. Although this endosperm requires the remaining enzymes in the pathway, it naturally accumulates GGPP. Further "Golden Rice" lines with only two foreign enzymes—bacterial phytoene desaturase and daffodil phytoene synthase—were developed because of the initial findings' publication **(Kobayashi et al., 2023)**.

There have also been reports of transgenic tomatoes that express phytoene synthase or phytoene desaturase. The overall amount of carotenoid content rose three times in the first scenario and up to four times in the second. According to **Black et al. (2023),** there were three times as much β-carotene present as usual.

8. HOST SELECTION

Proteins and nonproteins are the two main product categories produced by genetically modified cells (**Balabanova et al., 2015**). Encoding genes for proteins are inserted into plasmids or the host genome to produce protein products. These products

can be employed as industrial catalysts, human treatments, animal husbandry, or food processing. To manufacture nonprotein compounds, such as metabolites like amino acids, biofuels, antioxidants, and vitamins, genes encoding the necessary enzymes are introduced.

9. INVERSE METABOLIC ENGINEERING (IME)

Common metabolic engineering methods include using genetic cloning to increase the production of desired commodities and figuring out what controls speed. Beginning with a given phenotype, inverse metabolic engineering (IME) employs a global combinatorial technique to find genetic information explaining potential future performance enhancement options and furnishing valuable feedback knowledge **(Alseekh et al., 2023)**. The three steps of the IME method are as follows: (1) choose, generate, or compute a desired phenotypic; (2) determine the genetic information or environmental factors responsible for that phenotype; and (3) use evolutionary engineering to rebuild the genotype on an engineered strain. The enhanced capacity to explain a phenotype may be attributed in large part to one- and two-dimensional proteomics, mass spectrometry, quantitative real-time polymerase chain reaction, DNA sequencing, mRNA profiling technologies, mass spectrometry, and analytical methodologies. Through a variety of IME techniques, including overexpressed gene target identification, enrichment of the transformants displaying increased alcohol tolerance, and transformation using a Saccharomyces *cerevisiae* genomic library, **Zhang et al. (2022)** reported the discovery of four endogenous *S. cerevisiae* genes that confer enhanced alcohol tolerance. Several genes that confer better xylose-growing *S. cerevisiae* through IME were found by **(Patiño et al., 2019)**. Using two control strains and four recombinant *S. cerevisiae* strains with increased xylose growth, genome-wide transcription study was performed.

10. ENGINEERING OF SECRETORY PATHWAYS

Biopharmaceuticals are eager for heterologous protein products to secrete into the periplasm and media of eukaryotic and prokaryotic hosts, respectively. Secretion from the cytoplasm lowers production costs by greatly streamlining downstream purifying procedures. When proteins are overexpressed in the cytoplasm, it frequently results in insoluble clumps known as inclusion bodies. Inclusion bodies must be processed further, which takes time and money since it involves solubilizing and folding the proteins. Furthermore, there is a decreased likelihood of cytosolic protease activity and other protein species degrading and contaminating the released proteins. In their review of *Lactococcus lactis* protein secretion, (**Song et al. 2017** and **Ragab et al. 2020**) found that the synthesis of heterologous proteins was five times higher. By introducing five synonymous unusual codons in a specific region of hlyA, (**Pourhassan et al., 2022**) successfully generated an α-hemolysin (HlyA) hypersecretion strain via the hemolysin (type I) secretion route. The hyper secreter strain led to an eight-fold increase in HlyA secretion and demonstrated a 37% decrease in the expected translation rate of hlyA.

11 MODIFYING PLANT METABOLISM TO INTRODUCE NOVEL FEATURES INTO CROPS

Food security is a pressing issue given that there are currently 7 billion people on the planet. Harvesting crops with the best nutritional value possibly becomes increasingly important as land becomes scarcer. By upregulating these pathways, crops can become more nutritionally rich since many plant metabolites have significant positive effects on health and nutrition **(Hikal & Abdein, 2018** and **Lo'ay et al., 2021).** Important nutritional implications for terpenoid and phenylpropanoid chemicals in the human diet **(Han et al., 2023).**

12. FUTURE PERSPECTIVES

Numerous industrial methods, like the fabrication of chemicals and antibiotics, alternative energy sources, nutrient-enriched foods, and therapeutic protein synthesis, have seen a technological revolution thanks to metabolic engineering. In the last ten years, metabolic engineering applications have advanced dramatically to address human requirements in a sustainable manner by increasing production, cutting costs, and reducing pollutants. The development and acceptance of the newly formed discipline of synthetic biology will have a significant influence on the path of metabolic engineering, which has a very promising future. An engineering idea called "synthetic biology" aims to make it easier to carefully advance the engineering of biology for more creative and useful uses.

However, integration of systems biology with conventional bioengineering areas like as genetic, protein, and metabolic engineering is necessary before synthetic biology may be completely realized. Using whole-genome-scale data sets that are

collected, examined, and combined to create a quantitative phenotypic description of the system, systems biology is the quantitative study of biological systems. This method looks at the separate parts of the system; like the past applications of selection and random mutagenesis, metabolic engineering today requires a multimodal approach based on extensive screening studies and computational study of metabolic and regulatory networks. Cell and microbe research within the context of systems biology is therefore quite common.

The availability of biological information to measure physiology has enabled recent exponential breakthroughs in systems biology; nevertheless, future discovery and development will depend on the ability to examine high-throughput data sets with the right tools. To characterize biological networks, it is required to create mathematical ideas and map out regulators, proteins, RNAs, and other macromolecules. Protein interaction networks, metabolic networks, and regulatory networks must be created to create accurate mathematical models.

Synthetic biology is being facilitated by systems biology, which provides a deeper level of comprehension and a conceptual framework for comprehending life from fundamental principles. The de novo synthesis and design of novel proteins, genetic circuits, and metabolic networks will be required for the development of new biological systems.

Artemisinin, an antimalarial medication that was successfully manufactured in *E. coli* in the lab of synthetic biology pioneer Jay D. Keasling, is often sourced from an *Artemisia annua* plantation. To produce artificial cells that can operate according to human expectations, large multidisciplinary teams working on shared difficulties with comparable goals will need to collaborate.

More extensive uses of metabolic engineering ideas are starting to appear in the medical domain, as scientists must integrate patient, animal model, and tissue-culture data. When researching these conditions, it is beneficial to be able to integrate a large amount of data from studies on cellular metabolism since sugar metabolism and storage play a role in the development of diseases like diabetes and obesity. Further techniques that may be used on primary cells, entire tissues, or biological fluids include flow measurements, systems level modelling, and global profiling of transcript, metabolite, and protein stages. These procedures are essential for elucidating the relationships and roles of several subsystems. These techniques, which include molecular targets for novel medications and diagnostic markers, can be utilized to study the onset, course, and impact of disease therapy. The field of metabolic engineering, which is expanding quickly, will also help personalized medicine advance, as personalized medicine emphasizes the intentional use of specific patient data to optimize therapeutic and preventative therapy.

Recently, there has been a lot of interest focused on the development of medical terpenoid due to the important functions these molecules play in the prevention and treatment of many disorders. The need for these items develops as the prevalence of serious illnesses like cancer and malaria climbs globally, necessitating the rapid development of sustainable and alternative sources in place of natural and chemical ones, (**Ghazzawy et al. 2022**. Compared to conventional approaches, microbial fermentation of these unique chemicals offers a high yield, economical, and ecological process. The yeast has been demonstrated as a productive cell industrial unit for the large-scale synthesis of terpenoid compounds. Two successful instances of industrial manufacture are β-farnesene and artemisinic acid. To improve the TYP of therapeutic terpenoids and optimize metabolic engineering in this yeast, process optimization by evaluating several fermentation processes is required, as *S. cerevisiae* is unable to natively synthesize the target terpenoids. The commercialization of terpenoid applications is contingent upon the effective integration of fermentation process development with metabolic engineering. Process optimization may lower production costs and boost productivity by implementing effective downstream processing, physicochemical conditions, and medium composition improvements. The majority of the terpenoid titers that are acquired, mostly from batch fermentations, are at mg/L levels and have no practical significance. Conversely, fed-batch bioreactor fermentation is the most promising method for producing medicinal terpenoids in large quantities. At the g/L scale, it can yield great productivity and titers while lowering the product's toxicity. For some production scenarios, the pH and DO are important variables that influence the fermentation of terpenoids. While the ideal pH range for yeast development and high cell density is between 4 and 6, many terpenes may not dissolve in this pH range, which might hamper processing further on. But this may be resolved, for example, by adding base solution to harvest tanks at a high pH. One strategy to reduce the negative effects of terpenes is to use extraction agents during

production, albeit at additional cost. By removing products and optimizing the feed strategy in fed-batch fermentation, this issue may be resolved. In fed-batch operations, the DO is frequently utilized as a trigger to regulate yeast growth and product output. As low oxygen concentrations can reduce yeast capacity and excessive O_2 concentrations (conc.) can cause oxidative stress, which can be harmful to cells, optimal O_2 conc. are necessary to balance yeast health and productivity. In contrast, low temperatures cause high cell density to form later, while high temperatures have the potential to impair the vitality of cells. The proper oxygen concentration must be maintained for yeast to stay healthy and productive since too high of a concentration can cause oxidative stress, which can be harmful to cells, and too low of a concentration will reduce the potential of the yeast. Thus, it's critical to maintain a balance between yeast health and productivity. Minimum O_2 concentration can limit yeast's potential, while maximum concentration might induce oxidative stress, which can be detrimental to cells. In fact, the tendency is towards a rise in medicinal terpenoids produced via enhanced fermentation. The ability to introduce novel terpenoid biosynthesis pathways in *S. cerevisiae* using synthetic biology techniques and metabolic engineering advancements may open new possibilities to produce complex terpenes, like the popular "cannabinoids." The microbial fermentation of these incredibly uncommon and intriguing chemicals at a reasonable cost and high purity, independent of Cannabis plant development, may be of enormous advantage to the pharmaceutical business when the increased industrial demand for these molecules is anticipated.

13. CONCLUSION

Terpenoids are among the natural compounds with the widest variety. For several ailments, they provide important therapeutic benefits. These days, engaging the secondary metabolic pathway with biotic and abiotic elicitors is often the focus of increasing the synthesis of target terpenoids. The production of medicinal terpenoids can be controlled by a variety of plant metabolic engineering techniques that depend on Agrobacterium-mediated genetic transformation. Overexpressing genes linked to the terpenoids production pathway can improve the synthesis of therapeutic terpenoids in both homologous and ectopic plants. Furthermore, global regulation, which controls endogenous phytohormones, primary metabolism, and the relative transcription factors, may also significantly boost their production.

Plant metabolic engineering approaches have the potential to significantly enhance the amount of pharmaceutical terpenoids in medicinal plants. These terpenoids were substantially greater in transgenic plants than in control plants. Transgenic plants use the tumor inducing (Ti) plasmid to introduce specific DNA into the host genome. One advantage of growing these plants is that they yield a variety of terpenoids. To create therapeutic terpenoids, plant metabolic engineering has focused on discovering various TPSs, CYP450s, and glucosyl transferases as well as investigating the overexpression of transcription factors involved in the regulation of terpenoid biosynthesis. Future advancements in plant metabolic engineering will lead to the upregulation of an increasing number of valuable medicinal terpenoids. Therefore, we assume that all those tactics will raise the availability of rare medications, lower the cost of pricey medications, and raise people's standards of living.

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