

SOLANESOL PRODUCTION FROM NICOTIANA TABACUM CV. XANTHI CELL
SUSPENSION CULTURE AND PROCESS SCALE-UP



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SUSPENSION CULTURE AND PROCESS SCALE-UP

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ABSTRACT

SOLANESOL PRODUCTION FROM *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURE AND PROCESS SCALE-UP

Plants are important sources for wide range of different chemical compounds used in the production of fragrances, pigments, flavors, industrial feedstock, antimicrobials and pharmaceuticals. These chemicals are known as plant secondary metabolites which are generally responsible for the protection of the plant against environmental stress factors. Solanesol is a non-cyclic terpene found mainly in *Solanaceae* family plants and known to have highest accumulation in *Nicotiana tabacum* (tobacco) with 0.04-3.6 percent of leaf dry weight. It has antimicrobial, anti-cancer and anti-inflammatory effects that is mainly used as a precursor of Coenzyme Q-10. It is mainly extracted from the plants instead of chemical synthesis due to its long carbon chains. Secondary metabolites are generally extracted from whole plants which require large lands for growth, optimal environmental conditions and it is also labor intensive. Therefore, using plant cell suspension culture systems for the production of secondary metabolites has become one of the most important tools. In this study, it was aimed to achieve solanesol extraction from *Nicotiana tabacum* cv. Xanthi cell suspension culture and process scale-up of the suspension cultures. To our knowledge, this is the first study in the literature that focuses on the production of solanesol from tobacco cell suspension culture instead of whole tobacco plant or tobacco waste. *N. tabacum* cv. Xanthi callus cell lines were used to initiate the cell suspension culture which was maintained throughout the study. Optimal pH of the cell suspension cultures were determined as 6, cells were in log phase between days 1-11 and in stationary phase between days 11-15 according to the fresh weight, dry weight, pH and sugar consumption analysis. Optimal subculture period was determined as 7 to 9 days. Solanesol was extracted from *N. tabacum* cv. Xanthi cell suspension cultures for the first time in literature and had the average production of 14.56 ± 4.71 mg/ml which was the 1.46 percent of the dry weight. Scale-up parameters were optimized for growing plant cell suspension cultures in a stirred-tank bioreactor. This study will be the foundation for large-scale production of solanesol from *N. tabacum* cv. Xanthi cell suspension cultures.

ÖZET

NICOTIANA TABACUM CV. XANTHI HÜCRE SÜSPANSİYON KÜLTÜRÜNDEN SOLANESOL ÜRETİMİ VE ÖLÇEK BÜYÜTME ÇALIŞMALARI

Bitkiler, koku, pigment, aroma verici, endüstriyel hammadde, antimikrobiyal ve farmasötiklerin üretiminde kullanılan çok çeşitli kimyasal bileşikler için önemli kaynaklardır. Sekonder metabolit olarak bilinen bu kimyasallar, genel olarak bitkiyi çevresel stres faktörlerine karşı korurlar. Solanesol, esas olarak *Solanaceae* familyası bitkilerinde bulunan ve *Nicotiana tabacum*'da (tütün) en yüksek birikime, yaprak kuru ağırlığının yüzde 0.04-3.6'sını oluşturarak sahip olduğu bilinen halkasal yapıda olmayan terpendir. Antimikrobiyal, anti-kanser ve antienflamatuar etkileri vardır ve esas olarak Koenzim Q-10 öncüsü olarak kullanılır. Uzun karbon zincirleri nedeniyle solanesol genel olarak kimyasal sentez yerine bitkilerden ekstrakte edilir. Sekonder metabolitler genellikle bütün bitkilerden ekstrakte edilir ancak bu yöntemde bitki üretimi için geniş arazilere, optimal çevre koşullarına ve yüksek işgücüne ihtiyaç vardır. Bundan dolayı, sekonder metabolit üretimi için bitki hücre süspansiyon kültürü sistemlerini kullanmak önemli bir araç haline gelmiştir. Bu çalışmada, *Nicotiana tabacum* cv. Xanthi hücre süspansiyon kültüründen solanesol ekstraksiyonu ve süspansiyon kültürün ölçek büyütme çalışmalarının yapılması amaçlanmıştır. Bilgimiz dahilinde, bütün tütün bitkisi veya tütün atığı yerine tütün hücre süspansiyon kültüründen solanesol üretilmesine odaklanan literatürdeki ilk çalışmadır. *N. tabacum* cv. Xanthi kallus hücre hatları bitki hücre süspansiyon kültürünü başlatmak için kullanılmış ve çalışma boyunca kültürler devam ettirilmiştir. Yaş ağırlık, kuru ağırlık, pH ve şeker tüketim analizlerine göre hücre süspansiyon kültürlerinin optimal pH'nın 6 olduğu, hücrelerin 1-11. gün arasında log fazında ve 11-15. gün arasında durağan fazda oldukları belirlenmiştir. Optimal altkültür periyodu 7 ile 9 gün olarak belirlenmiştir. Solanesol literatürde ilk kez *N. tabacum* cv. Xanthi hücre süspansiyon kültüründen ekstrakte edilmiştir. Solanesol üretimi, ortalama 14.56 ± 4.71 mg/ml ile kuru ağırlığın yüzde 1.46'sı kadardır. Ölçek büyütme parametreleri, bitki hücre süspansiyon kültürlerini karıştırılmalı tanklı biyoreaktörde büyütebilmek için optimize edilmiştir. Bu çalışma, büyük ölçekte *N. tabacum* cv. Xanthi hücre süspansiyon kültürlerinden solanesol üretimi için temel oluşturacaktır.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	V
ÖZET	VI
LIST OF FIGURES	X
LIST OF TABLES.....	XIII
LIST OF SYMBOLS/ABBREVIATIONS.....	XIV
1. INTRODUCTION.....	1
2. LITERATURE REVIEW	3
2.1. PLANT TISSUE CULTURE.....	3
2.2. PLANT METABOLITES	5
2.2.1. Primary Metabolism in Plants.....	5
2.2.2. Secondary Metabolism in Plants.....	5
2.3. CLASSICAL AND ADVANCED TECHNIQUES FOR SECONDARY METABOLITE PRODUCTION IN PLANT TISSUE CULTURE	9
2.3.1. Classical Techniques for Secondary Metabolite Production in Plant Tissue Culture.....	10
2.3.1.1. <i>Cell and Organ Cultures</i>	10
2.3.1.2. <i>Somatic Embryogenesis</i>	12
2.3.1.3. <i>Strain Improvement</i>	13
2.3.1.4. <i>Media Optimization</i>	13
2.3.2. Advanced Techniques for Secondary Metabolite Production in Plant Tissue Culture...	15
2.3.2.1. <i>Elicitation</i>	15
2.3.2.2. <i>Precursor Feeding</i>	16
2.3.2.3. <i>Biotransformation</i>	16
2.3.2.4. <i>Cell Immobilization</i>	16
2.3.2.5. <i>Genetic Engineering</i>	17
2.3.2.6. <i>Use of Endophytes</i>	17
2.3.2.7. <i>Molecular Pharming</i>	17

2.3.2.8. Scale-up Techniques	17
2.4. NICOTIANA TABACUM AND ITS SECONDARY METABOLITES	18
2.4.1. Secondary Metabolites of <i>Nicotiana tabacum</i>	19
2.4.2. Solanesol and Its Biosynthesis	20
2.5. AIM OF THE STUDY	26
3. MATERIALS	27
3.1. CHEMICALS	27
3.2. GLASSWARE & CONSUMABLES	27
3.3. EQUIPMENT	28
3.4. PLANT MATERIAL	28
4. METHODS	29
4.1. MEDIA COMPOSITION	29
4.2. CALLUS INDUCTION AND CELL LINE MAINTENANCE OF <i>NICOTIANA TABACUM</i> CV. XANTHI	29
4.3. MAINTENANCE OF STABLE <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURE	30
4.4. DETERMINATION OF CELL VIABILITY OF <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURE	31
4.5. CONSTRUCTION OF GROWTH CURVE FOR <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURE	31
4.6. EXTRACTION OF SOLANESOL FROM <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURE	32
4.7. HPLC ANALYSIS FOR THE QUANTIFICATION OF SOLANESOL	32
4.8. PARAMETER OPTIMIZATION FOR <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURE SCALE-UP	33
4.9. STATISTICAL ANALYSIS	34
5. RESULTS AND DISCUSSION	35
5.1. CALLUS INDUCTION AND MAINTENANCE OF CELL LINES	35
5.2. MAINTENANCE OF <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURES	37

5.3. GROWTH CURVE ANALYSIS OF <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURES	42
5.4. EXTRACTION AND ANALYSIS OF SOLANESOL FROM <i>NICOTIANA TABACUM</i> CV. XANTI CELL SUSPENSION CULTURES	46
5.5. PROCESS SCALE-UP OF <i>NICOTIANA TABACUM</i> CV. XANTHI CELL SUSPENSION CULTURE VIA STIRRED-TANK BIOREACTOR	51
6. CONCLUSION AND FUTURE PROSPECTS	58
REFERENCES	60
APPENDIX A.....	76
APPENDIX B.....	78

LIST OF FIGURES

Figure 2.1. Various application areas of plant tissue culture [19]	4
Figure 2.2. Workflow of plant cell suspension culture and scale-up studies [73].....	12
Figure 2.3. Compositions of two commonly used PTC media [71]	14
Figure 2.4. Different types of elicitors and their effects on SM production [85–88]	15
Figure 2.5. <i>Nicotiana tabacum</i> cv. Xanthi grown in greenhouse conditions.....	19
Figure 2.6. Primary and secondary metabolite biosynthesis pathways [103].....	20
Figure 2.7. Chemical structure of solanesol [107].....	21
Figure 2.8. Solanesol biosynthesis in plants [103,107]	23
Figure 5.1. <i>Nicotiana tabacum</i> cv. Xanthi leaf cell lines maintained in NtCIM3 media and used throughout the study	35
Figure 5.2. <i>Nicotiana tabacum</i> cv. Xanthi in NtGM2 media (a) after seed germination, (b) before callus induction, (c) before starting micropropagation.....	36
Figure 5.3. <i>Nicotiana tabacum</i> cv. Xanthi callus induction on NtCIM3 media (a) explants taken from the leaves for induction, (b) start of callus formation, (c) calli before transferring to plant growth room, and (d) calli in plant growth room	36
Figure 5.4. <i>Nicotiana tabacum</i> cv. Xanthi micropropagations in MS1BACH media (a) one week after subculture, and (b) 4 weeks after subculture.....	37
Figure 5.5. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension cultures in NtCIM5 media at 18 th subculture (a) after subculture, and (b) before subculture	38
Figure 5.6. Cell suspension culture subculturing setup inside the biosafety cabinet.....	38
Figure 5.7. Stereo microscope image of <i>N. tabacum</i> cv. Xanthi cluster cell intersect with 32x magnification	39

Figure 5.8. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension cultures growth rate changes throughout 21 subcultures of maintenance	40
Figure 5.9. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension light microscope images (a) 4x magnification, and (b) 10x magnification.....	41
Figure 5.10. Viability test applied to <i>N. tabacum</i> cv. Xanthi cell suspension cultures showing living cells with bright green color under fluorescent microscope with FITC filter	41
Figure 5.11. Fresh weight changes of <i>N. tabacum</i> cv. Xanthi cell suspension cultures in 15 days growth period.....	43
Figure 5.12. Dry weight changes of <i>N. tabacum</i> cv. Xanthi cell suspension cultures in 15 days growth period.....	44
Figure 5.13. pH changes of <i>N. tabacum</i> cv. Xanthi cell suspension cultures in 15 days growth period	45
Figure 5.14. Correlation between FW, DW and pH in <i>N. tabacum</i> cv. Xanthi cell suspension cultures in 15 days growth period.....	45
Figure 5.15. Sugar consumption of <i>N. tabacum</i> cv. Xanthi cell suspension cultures throughout 15 days growth period	46
Figure 5.16. HPLC chromatogram of solanesol standards	47
Figure 5.17. Solanesol concentration vs. peak area, calibration curve and equation	47
Figure 5.18. HPLC chromatogram of solanesol extraction from <i>N. tabacum</i> cv. Xanthi cells (*:solaneseol peaks).....	49
Figure 5.19. Detailed HPLC chromatogram of solanesol extracted from <i>N. tabacum</i> cv. Xanthi cells showing each replicate with standards	49
Figure 5.20. HPLC chromatogram of solanesol extraction from dry tobacco leaves.....	51
Figure 5.21. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 1 at (a) day 0, (b) day 1, and (c) day 2	52

Figure 5.22. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 1 sugar concentrations in day 1 and 2	53
Figure 5.23. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 2 at (a) day 0, (b) day 1, (c) day 2, (d) day 3, and (e) day 7.....	53
Figure 5.24. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 2 sugar concentration changes in 7 days	54
Figure 5.25. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 3 at (a) day 0, (b) day 1, (c) day 2, and (d) day 3.....	55
Figure 5.26. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 3 sugar concentration changes in 3 days	55
Figure 5.27. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 4 at (a) day 0, (b) day 1, (c) day 3, (d) day 5, and (e) day 7.....	56
Figure 5.28. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 4 sugar concentration changes in 7 days	56
Figure 5.29. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension culture bioreactor trial 5 at (a) day 0, (b) day 1, (c) day 3, (d) day 5, and (e) day 6.....	57

LIST OF TABLES

Table 4.1. <i>Nicotiana tabacum</i> cv. Xanthi media compositions used in this study	29
Table 4.2. Parameter changes in <i>N. tabacum</i> cv. Xanthi scale-up studies.....	33
Table 5.1. <i>Nicotiana tabacum</i> cv. Xanthi cell suspension cultures used for the construction of growth curve with three biological replicates	42
Table 5.2. Retention time and peak area for solanesol standards	47
Table 5.3. Retention time, peak area and concentration (mg/ml) of solanesol extraction from <i>N. tabacum</i> cv. Xanthi cells	50
Table 5.4. Effect of parameter changes in <i>N. tabacum</i> cv. Xanthi final cell mass in scale-up studies	52

LIST OF SYMBOLS/ ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ATP	Adenosine triphosphate
BAP	6-Benzylaminopurine
DMAPP	Dimethylallyl diphosphate
DW	Dry weight
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
FDA	Flourescein diacetate
FPP	Farnesyl diphosphate
FW	Fresh weight
GAP	Glyceraldehyde-3-phosphate
GC	Gas chromatography
GGPP	Geranyl geranyl diphosphate
GPS	Geranyl diphosphate
HPLC	High performance liquid chromatography
IPI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl diphosphate
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
LS	Linsmaier and Skoog
MEP	2-C-methyl-D-erythritol 4-phosphate
MS	Murashige and Skoog
MS1BACH	<i>Nicotiana tabacum</i> cv. Xanthi micropropagation media
MVA	Mevalonate
NAA	1-Naphtaleneacetic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NtCIM3	<i>Nicotiana tabacum</i> cv. Xanthi callus induction media
NtCIM5	<i>Nicotiana tabacum</i> cv. Xanthi suspension media
NtGM2	<i>Nicotiana tabacum</i> cv. Xanthi germination media

PCSC	Plant cell suspension culture
PGR	Plant growth regulator
PTC	Plant tissue culture
R	Replicate
RP-HPLC	Reversed phased high performance liquid chromatography
RT	Retention time
SDB	N-solanesyl-N,N'-bis(3,4-dimethoxybenzyl) ethylenediamine
SM	Secondary metabolite
SPE	Solid-liquid extraction
SPP	Solanesyl diphosphate
SPS	Solanesyl diphosphate synthase
ROS	Reactive oxygen species
TLC	Thin layer chromatography
UPLC	Ultra-performance liquid chromatography
vvm	Gas flow rate per reactor volume

1. INTRODUCTION

Plants are important sources for wide range of chemical compounds including fragrances, pigments, flavors, industrial feedstock, antimicrobials and pharmaceuticals [1]. These chemicals produced by plants are called plant secondary metabolites. Secondary metabolites are the products of primary metabolism of plant cells that are not involved in growth, development, reproduction and generation of biomass [2]. Secondary metabolites are specific to plant species and their concentration differs even among individual plants in the same population. Secondary metabolites are responsible for the protection of plants against biotic and abiotic stress inducers such as insects, animals, high temperature, UV irradiation and viruses [3]. Until the isolation of morphine from opium poppy in 19th century by Friedrich Wilhelm, secondary metabolites were seen as the waste of primary metabolism. Since then, there have been extensive studies on their extraction, purification and large-scale production. Generally, these metabolites are extracted from whole plants or synthesized synthetically by chemical reactions [4]. However, using whole plants for secondary metabolite production comes with its consequences. Plants require large arable lands and, optimal conditions for growth and intensive labor work as well. Besides, downstream processes of these plant derived secondary metabolites are complex and expensive. In order to produce large amounts of secondary metabolites for commercial use, using plant cell suspension cultures rather than whole plants are the focus of attention [5].

Nicotiana tabacum (tobacco) is an economically important perennial plant that is commercially used for tobacco production throughout the world. In the 16th century, its medicinal properties started to be discovered through diverse studies on tobacco plants [6]. *N. tabacum* produces wide range of secondary metabolites which have been extensively studied including major groups like isoprenoids, alkaloids and flavonoids. More than 2500 phytochemical compounds of *N. tabacum* have been identified with these studies. Nicotine is the most known alkaloid that is produced in tobacco leaves [7].

Solanesol is a secondary metabolite that is produced by plants in Solanaceae family. It is an isoprenoid alcohol abundantly found in tobacco leaves [8]. Its production in tobacco is mainly dependent on the genotype and environmental factors [9]. Solanesol have anti-

microbial, anti-cancer, anti-ulcer, and anti-inflammatory activities in addition to its importance as pharmaceutical intermediates for coenzyme Q10, vitamin K2 and SDB (N-solanesyl-N,N'-bis(3,4-dimethoxybenzyl) ethylenediamine) [10]. Solanesol concentration ranges from 0.4 to 4 percent in dry leaf weight [11]. Solanesol is generally extracted from whole tobacco plant and tobacco waste. High purity solanesol has a growing international demand that is estimated to reach 8000 to 10000 tons per year since it is mostly used for coenzyme Q10 production in pharmaceutical industry. According to the data provided by Food and Agriculture Organization of United States (FAO), China is the main producer of tobacco with 42 percent with the largest share in solanesol production as well [12].

In this study, extraction of solanesol from *Nicotiana tabacum* cv. Xanthi cell suspension culture and process scale-up of cell suspension cultures were aimed. To our knowledge, this is the first study that focuses on the extraction of solanesol from plant cell suspension culture instead of extracting from whole plant or industrial waste. In order to achieve this goal, *N. tabacum* cv. Xanthi cell suspension cultures were initiated from cell lines and maintained for 21 subcultures. Growth parameters were analyzed during the maintenance period by regularly measuring fresh weight, dry weight, pH and sugar consumption of *N. tabacum* cv. Xanthi cells suspension cultures for 15 days. After that, solanesol was extracted from *N. tabacum* cv. Xanthi cell suspension culture biomass and analyzed with RP-HPLC (Reversed phased high performance liquid chromatography). Finally, bioreactor parameter optimization studies of *N. tabacum* cv. Xanthi cell suspension culture for process scale-up were done with the use of a stirred-tank bioreactor.

2. LITERATURE REVIEW

2.1. PLANT TISSUE CULTURE

Plant tissue culture (PTC) is the *in vitro* aseptic culture of whole plants, organs, tissues, cells and their components under controlled physical and chemical conditions. It has commercial applications while being an important tool for basic and applied studies [13]. The basis of plant tissue culture comes from the discovery of cell and cell theory. In 1902, Gottlieb Haberlandt established the theoretical basis of plant tissue culture and concept of totipotency by his experiments on the culture of single palisade cells from leaves in Knop's salt solution enriched with sucrose [14]. He was unable to keep cells alive more than a month; however, he laid down the foundation of plant tissue culture that made him the father of plant tissue culture.

In PTC, plant organs and cells are grown *in vitro* under controlled an aseptic environment on an artificial media. PTC medium is crucial for the growth and development of plants since it contains all the nutrients required. The main components of PTC medium are macronutrients, micronutrients, vitamins, other organic components, carbon source, plant growth regulators and some solidifying agents in case of a solid medium. In 1962, Murashige and Skoog medium (MS medium) was developed by examining tobacco callus ashes and it has become the most extensively used medium for the *in vitro* propagation of many different plant species [15]. The pH of the media should be between 5.2-5.8 since it affects both the activity of plant growth regulators and plant physiology. Each components of the medium have particular effects depending on the plant species.

PTC medium includes macronutrients such as nitrogen (N), magnesium (Mg), potassium (K), calcium (Ca), phosphorous (P) and sulfur (S) which are relatively in high amounts. The other important components of basal salt medium are micronutrients such as manganese (Mn), iron (Fe), boron (B), zinc (Zn), copper (Cu), molybdenum (Mo) and cobalt (Co) which are found in smaller amounts. Vitamins are also required for a healthy growth of PTC. The most common vitamins used in PTC are thiamine (vitamin B₁), nicotinic acid and pyridoxine (B₆). In addition to these, plants require an external carbon source which is generally in the form of sugars. The most commonly used carbon source in PTC is sucrose; however,

according to the species used and experimental setup glucose, sorbitol and maltose can be used as carbon source [1].

Plant growth regulators (PGR's) such as auxins, cytokinins, giberellins, ethylene and abscisic acid are the most commonly used PGR's that plays essential roles in determining the growth and development pathways of plant cells. Auxins and cytokinins are the most common PGR's types and concentrations used in PTC's are dependent on the plant species [16]. Finally, the medium used in PTC are generally solidified with agar, gelrite or phytigel which provides a support for the plant cells and tissues during growth.

There are two main types of plant tissue culture which are cultures of organized structures and cultures of unorganized cells. Creation or maintenance of a defined structure comes from the organized growth that can be seen in higher plants. Cultures of isolated plant organs such as leaf culture, root culture and embryo culture are the examples to cultures of organized structure [17]. Callus culture, cell suspension culture, protoplast culture and immobilized cell cultures are defined as cultures of unorganized cells [18]. Basic, economic and commercial application of PTC can be seen in Figure 2.1.

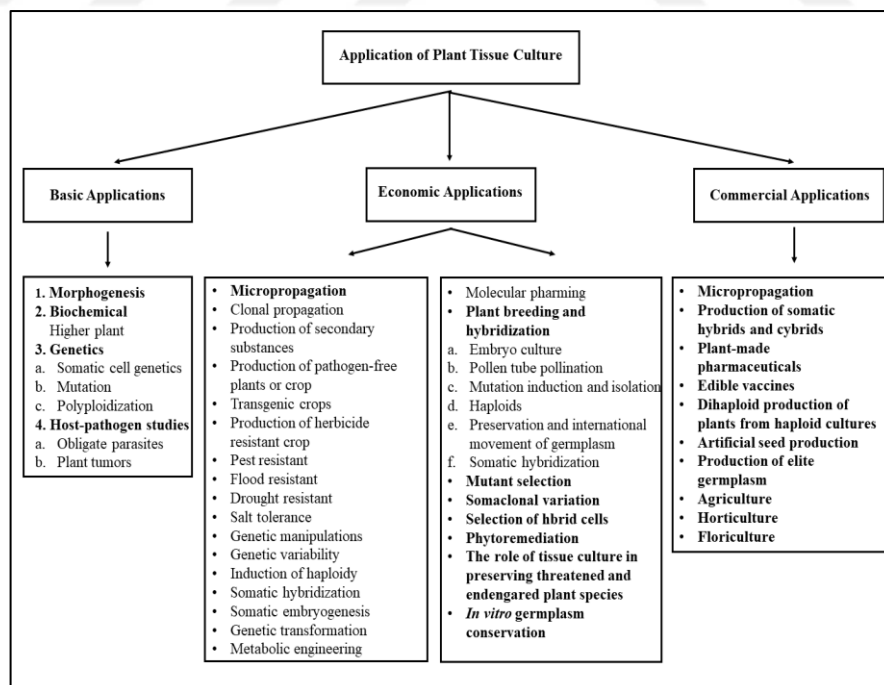


Figure 2.1. Various application areas of plant tissue culture [19]

2.2. PLANT METABOLITES

Low molecular weight organic compounds that are synthesized from inorganic precursors that plays an important role in cellular activities are called metabolites [20]. Plants are the sources of approximately 200.000 to 1 million metabolites that are functionally and structurally different than each other. They are generally responsible for plant growth, development and adaptation mechanism due to environmental changes [21]. Metabolites also play an important role in stress resistance and tolerance mechanisms of plants. Although they are produced inside the cell, they can work both in intracellular and extracellular activities [22]. Plant metabolites are generally divided into three groups according to their functions which are primary metabolites, secondary metabolites and hormones. However, there is not a clear line between these groups and metabolites are generally biochemically interchangeable with metabolite modifications inside the cell [23]. Information about plant metabolites are very limited and in some cases non-existing due to unknown plant biosynthesis pathways and/or instant changes of metabolite levels in plants according to stress factors [20].

2.2.1. Primary Metabolism in Plants

Basic life functions such as growth, development, reproduction and storage are classified as primary metabolism [22]. Glycolysis, Krebs cycle and photosynthesis components like sugars, lipids, amino acids, ethanol, ATP and Acetyl-CoA are the most abundantly found primary metabolites [24]. Primary metabolites are generally present in many different organisms with the same structures. They are found in the plants in higher concentrations when compared with secondary metabolites and can act as precursors for the production of secondary metabolites [25].

2.2.2. Secondary Metabolism in Plants

The organic compounds which are not responsible for growth, development or reproduction directly are referred as secondary metabolites. Secondary metabolites (SMs) are generally responsible from the defense mechanism of the plants against biotic and abiotic stress. Since

plants are sessile organisms, they require an effective defense mechanism against environmental stress factors in order to survive [21]. Until recently, SMs were classified as the waste of primary metabolism; however, it has been shown that they increase the survival chance of plants by improving growth and development under different stress factors. This shows that SMs can act like important primary metabolites [26]. SMs are responsible from signaling, regulation of primary metabolism and inducing plant characteristics (color, fragrance, form etc.) according to environmental stress factors. Production of color and fragrance can be examples of SMs. They help plants to attract pollinators while creating a defense against animals. It is estimated that plant kingdom produces 200.000 to 1 million different metabolites and approximately 100.000 of them are SMs that are specific to taxonomic groups [27,28].

Biosynthesis pathways of both primary and secondary metabolites follow one another. Since biosynthetic reactions requires energy, it starts with the glycolysis of carbohydrates and krebs cycle. ATP (adenosine triphosphate) is formed through oxidation of glucose, fatty acids and amino acids (catabolism of primary compounds). ATP is used as a fuel in anabolic reactions that also include intermediate molecules on the pathways. This cycle of anabolism and catabolism requires the presence of reducing agents or hydrogen donors like NADP (nicotinamide adenine dinucleotide phosphate) and catalysts like coenzyme A [29]. Commonly used pathways for the biosynthesis of SMs are shikimic acid pathway for aromatic alkaloids, phenols and tannins; pentose phosphate pathway for glycosides and polysaccharides; mevalonic acid and methylerythritol phosphate pathway for terpenes, alkaloids and steroids [30].

SMs have different classifications so that they can be classified based on their biosynthetic pathways or composition (nitrogen-containing compounds, phenolics and terpenes), structures (polyketides, isoprenoids, alkaloids, flavonoids and phenylpropanoids) and structural modifications of common carbon backbone (nitrogen-containing, benzenoids, flavonoids, terpenes and phenylpropanoids) [23,31,32]. SMs are generally classified based on their biosynthetic pathways of three major groups includes terpenes, alkaloids and phenolics. According to the specific structures tannins, saponins and glycosides can be a part of them [33].

First and largest group of SMs in plants are terpenes. There are more than 40.000 different molecules in this group. They are also known as isoprenoids and classified according to the

number of isoprenoid units (five-carbon unit) that they consist of such as hemiterpenes (C_5), triterpenes (six C_5 units) and polyterpenes (n-number of C_5 units) [34]. Plants like lemon, mint and ginger generally contains terpenes in their flowers or fruits as a mixture of volatile compounds and fragrance. Terpenes have a wide range of biological functions since they participate in both primary and secondary metabolism. In the primary metabolism, they are working as photosynthetic pigments (carotenoids), electron carriers (plastoquinone and ubiquinone), plant growth regulators (giberellins, brassinosteroids, stringolactones, abscisic acid) and cell membrane parts such as phytosterols whereas in secondary metabolism they are working as defense molecules against animals and insects or molecules responsible for attracting pollinators [35].

Terpene (isoprenoids) biosynthesis in plants involves two pathways which are mevalonate (MVA) pathway in cytosol and methylerythritol phosphate (MEP) pathway in plastids. Isopentenyl diphosphate is involved in the synthesis of sterols, brassinosteroids, polyphenols, sesquiterpenes and it is provided by MVA pathway whereas precursors of carotenoids, mono- and diterpenes, giberellins, chlorophylls and phytohormones are provided by the MEP pathway. Isoprenoids are generally insoluble in water and biosynthesis of these compounds from primary metabolites starts with the formation of isopentenyl diphosphate (IPP) from acetyl-CoA either from MVA or MEP pathways [34,36].

Studies on terpenes revealed that they have many different pharmacological and physiological properties. They became valuable sources for medicinal and industrial applications. Squalene (UV protector) isolated from olive oil, farnesol (fragrance molecule) isolated from *Citrus aurantium*, rubber (restorative material) isolated from *Palaquim gutta* and vitamin E (antioxidant) isolated from *Corylus avellana* L. can be examples of different applications of terpenes [37–40].

Alkaloids are also a large secondary metabolite group that contains nitrogen atom in their structure. They are found in plant, bacteria, fungi and animals. However, they are primarily isolated from plants. Plants produce different alkaloids together where one of them dominates the others. Biosynthetic origin of alkaloids is common in all plants even if they have slight structural differences. Alkaloid concentration varies even in different parts of the same plant. They are mostly toxic compounds that responds to common precipitation reactions [41,42]. Until recently, alkaloids were considered as waste of nitrogen metabolism since they are not vital for plants. However, they are found to play a key role in the defense

mechanism of plants due to their toxicity. High toxicity protects plants from animals and microorganism while helping plants to compete other species in the same habitat [43].

Alkaloids are generally classified according to their biosynthetic origin which are true (or pure) alkaloids, protoalkaloids and pseudoalkaloids. Majority of plant alkaloids are true alkaloids containing intracyclic nitrogen which is highly reactive even in small quantities. Precursor molecules of alkaloids are amino acids (L-tyrosine, L-histidine, L-lysine and L-arginine) and they are generally found as salts [44]. When nitrogen group is not a part of heterocycle these are called protoalkaloids. L-tryptophan and L-ornitine are their precursor molecules and they are also considered as aromatic amines. Pseudoalkaloids are not derived from amino acid; however, they contain heterocyclic ring with nitrogen [45,46].

When compared with other secondary metabolite classes, alkaloids have unique physiological and toxicological properties that make them medically useful since they directly affect the central nervous system [33]. There are more than 15,000 alkaloids isolated from plants. Nicotine (stimulant and anti-herbivore) isolated from *Solanaceae* family, morphine (anesthetic and narcotic) isolated from *Papaveraceae* family, mescaline (hallucinogen) isolated from *Cactaceae* family, berberine (anti-bacterial/viral and anti-diabetic) isolated from *Berberidaceae* family can be examples of alkaloids [47–49].

Phenolics are one of the main classes of SMs in plants that contains hydroxyl groups directly attached to aromatic hydrocarbons in their structures. This class includes more than 10,000 compounds [50]. They are a diverse group of SMs classified according to the number of carbons present which are simple phenols, phenolic acids, acetophenones, cinnamic acids, coumarins, flavonoids, biflavonyls, benzophenones, stilbenes, quinones and betacyanins [51,52]. Biosynthesis of phenolics in plants uses two pathways which are shikimic acid pathway and MVA pathway. MVA pathway provides the synthesis of quinones and xanthenes whereas shikimic acid pathway provides cinnamic acids, phenylalanine and its derivatives. Synthesis of flavonoids uses precursors of both shikimic acid and MVA pathways [53–55].

Phenolics have wide range of functions like acting as antioxidants due to their quick oxidation properties. They accumulate in seeds to protect embryo and also on the surface of the leaves to capture UV radiation [2,56]. They are responsible for the color and fragrance of the plants making them appealing to pollinators while generating unappealing textures for

animals [57]. Resveratrol (antioxidant) isolated from *Viteceae* family, caffeic acid (antioxidant and anti-inflammatory) isolated from *Coffea arabica L.* and cyanidin (pigment) isolated from *Viteceae* and *Rosaceae* families can be examples of phenolics [55].

2.3. CLASSICAL AND ADVANCED TECHNIQUES FOR SECONDARY METABOLITE PRODUCTION IN PLANT TISSUE CULTURE

Cultivation of the plants in their natural habitat in order to produce secondary metabolites is time consuming, labor intensive and can be relatively insufficient when compared with *in vitro* cultivation of plants as cell, tissue or organ cultures. Especially for the production of pharmaceutically important SMs, plant tissue culture has many different advantages such as rapid production, constant availability of the consistent amount of product not depending on climate, location, not requiring herbicides or pesticides and production of high-purity and high-quality products in large amounts [58].

SMs are produced in PTC with two stage system as accumulation of biomass and synthesis of the secondary metabolite. The steps generally includes, selection and screening of the plant in order to choose the best genotype, callus initiation from the explants, subculturing until homogenous and genetically stable cells are achieved (cell line), screening the cell lines to choose the one with highest productivity, cell suspension studies and bioreactor studies [59,60]. There are classical and advanced techniques used in PTC in order to achieve higher amounts of SMs. Classical techniques include; 1) cell and organ cultures, 2) somatic embryogenesis, 3) strain improvements via selection or screening and 4) optimization of the media and culture conditions. On the other hand, advanced techniques include; 1) elicitation, 2) precursor feeding, 3) biotransformation, 4) cell immobilization, 5) genetic engineering, 6) endophytes, 7) molecular pharming, 8) scale-up techniques [61].

2.3.1. Classical Techniques for Secondary Metabolite Production in Plant Tissue Culture

2.3.1.1. Cell and Organ Cultures

Cell and organ cultures are formed by taking explants from specific organs (leaf, shoot, node, root etc.) of a plant and culturing *in vitro*. Studies on organ cultures started in the beginning of 20th century and accelerated after the development of MS medium in 1962 [62]. Supplementing the culture medium with hormones affected the growth and development of the cultures which lead to an increase in the localized production of SMs [63]. Using cell and organ cultures have some advantages like rapid production, having controlled conditions to produce higher amounts of SMs, production of the organ specific metabolites easily, creating a base for advanced techniques to be used (elicitation, immobilization etc.) and allowing scale-up technologies to be used to improve the yield [64].

Plant cell suspension cultures (PCSCs) are important tools in secondary metabolite production studies. After the isolation of a single plant cell in 1902, studies on plant cells lead to the production of first plant cell suspension in 1958 by Muir [65]. PCSCs are *in vitro* culture of dedifferentiated plant cells (callus) in a liquid medium. PCSCs have three main steps which are initiation, maintenance and scale-up.

Aseptically inoculating sterile liquid medium with friable calli in a closed environment (flasks) is called initiation of PCSCs. It starts with the initiation of callus cultures which is achieved by sterilizing tissues or seeds and inducing callus formation via different media compositions. After callus formation is achieved, calli are subcultured in periods (every 2-6 weeks depending on the species) into a fresh solid-medium and cells that have higher SM yield are selected in meantime. Obtaining a stable, homogenous, high-yielding cell line is important for the initiation of suspension cultures. This process of inducing callus formation to initiation of suspension culture can take 6-9 months [66].

Calli that have been selected for suspension studies are generally cut into small pieces and placed in a liquid medium inside a flask which is the initiation process of PCSCs. Flasks that contain the PCSCs are generally kept in orbital shakers with a specific temperature and rpm depending on the species. After initiating the suspension culture, cells have to be subcultured

regularly (every 5-15 days depending on the cells) to obtain fine and stable cell suspension culture. Plant cells have a tendency to produce aggregates due to their cell wall structures which is generally required for cell growth and production of SMs in small levels. However, obtaining fine cell suspension culture is important for stable growth with higher SM production. In order to achieve a fine suspension culture, techniques like pipetting, filtering and addition of pectinase are generally used [67,68].

After obtaining a fine cell suspension culture, maintenance step starts by regularly subculturing cells. In order to start SM production studies on suspension cultures, PCSCs need to become stabilized homogenous cultures. Stabilization period depends on the species and subculturing techniques that are used. There are three different techniques for subculturing cell suspensions such as weighing, pipetting and pouring. In the weighing technique, suspension cultures are filtered through a vacuum filtration system and the cell mass is weighed before subculturing into fresh medium. Certain cell weight is weighed for a specific medium volume which results in a similar cell density in every subculture. This technique has a high reproducibility with high contamination risk. In pipetting technique, a certain volume of cell suspension is taken and inoculated in a fresh medium. This technique generally results in a nonhomogenous cells suspension culture since it requires constant shaking while taking cells with a pipette. Last technique is pouring which is the addition of certain volume of fresh medium onto cell suspension culture and dividing it into more flasks. This is a much simpler technique when compared with other two; however, it has low reproducibility. Choosing the subculturing technique depends on the cell type and precision of the study. High reproducibility is important in SM production studies so the weighing method is generally used in these studies. Subculturing period for PCSCs are determined by the growth cycles of the cells. In order to obtain high amounts of biomass, cell suspensions should be subcultured at the end of their logarithmic phase (log phase). Suspension cultures are generally subcultured before post-stationary phase since it results in a brown color due to oxidation and increases the amount of dead cells [66–70].

Scale-up studies generally starts after obtaining homogenous fine cell suspension culture by transferring the suspension cells from 100-250 ml flasks to 500-2000 ml flasks. After cells are acclimated into higher volume, they are transferred into lab scale bioreactors which are 1-10 L. Once growth parameters (temperature, light intensity, pH etc.) are optimized they can be transferred into larger bioreactors (50-1000 L) for commercial production. These

procedures are very important especially for the production of pharmaceutically important SMs [71,72]. Workflow of PCSCs and scale-up studies can be seen in Figure 2.2.

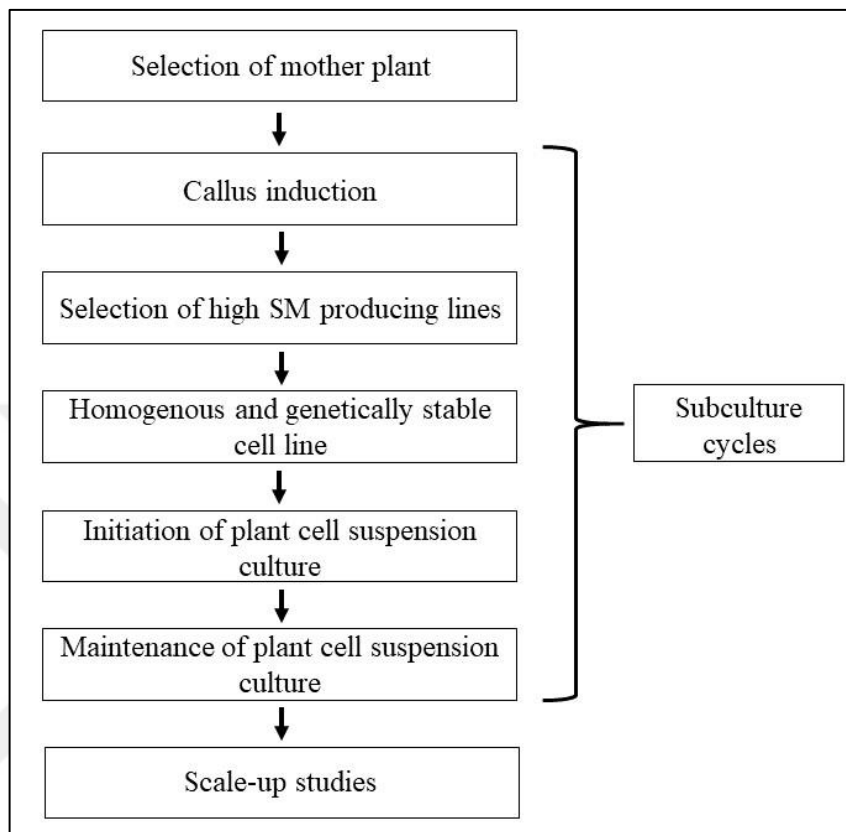


Figure 2.2. Workflow of plant cell suspension culture and scale-up studies [73]

2.3.1.2. *Somatic Embryogenesis*

Somatic embryogenesis is a term that defines the embryo production from somatic cells. When somatic embryogenesis is induced on the explants taken from the plant, it is referred as direct embryogenesis whereas in indirect embryogenesis, induction is applied on to unorganized cells (callus) [74]. This technique is especially important for increasing the yield of SMs with genetic and metabolic engineering. Studies showed that SM accumulation on somatic embryos were higher than zygotic embryos such as rosmarinic acid production from *Ocimum sanctum* L. somatic embryogenic callus [75].

2.3.1.3. Strain Improvement

Strain improvement is generally defined for microorganism which is the enhancement of a specific trait in order to produce it in large amounts. Enhancing the SM production through screening and selection can be the definition of strain improvement in PTC. In strain improvement firstly highest SM producing mother plant is determined via different selection techniques and callus is induced and subcultured frequently [62,76]. Somaclonal variations and epigenetic instability can cause problems in this stage; however, they can be overcome by optimizing media, hormones and environmental conditions. Secondly, cloned cells are screened with different techniques (visual screening, chemical assays, HPLC etc.) and can be induced for higher SM production by inducing mutagenesis or application of elicitors [77].

2.3.1.4. Media Optimization

Media optimization is one of the most important techniques in PTC with the optimization of culture conditions. Media components, hormones, temperature, pH and light intensity effects the production of SMs since they directly affect the pathways responsible for both biomass accumulation and SM production. There are many different types of PTC media including widely used Murashige and Skoog (MS), Gamborg (B5) and Linsmaier and Skoog (LS) with changing macro and microelement compositions (Figure 2.3.) [71,78]. Main carbon source of plant cells is sugar (sucrose, glucose or fructose) which is generally supplemented into the medium as sucrose. Sucrose also acts as osmotic agent and increased sucrose concentrations from the optimal ranges create high osmotic pressure which can repress the growth and SM production of plant cells. Generally optimum sucrose concentration for plant cells is between 2 to 4 percent [79]. In order to produce high amounts of alkaloids from *Catharanthus roseus*, 8 percent sucrose was used. On the other hand, using 3 percent sucrose on *Vitis vinifera* cultures was effective to increase the anthocyanin production [80,81].

	Murashige and Skoog (MS)	Gamborg (B5)
Macroelements (mg/L)		
NH₄NO₃	1.65	-
KNO₃	1.90	2.50
CaCl₂·2H₂O	440	150
MgSO₄·7H₂O	370	250
KH₂PO₄	170	-
(NH₄)₂SO₄	-	134
NaH₂PO₄·H₂O	-	150
Microelements (mg/L)		
FeSO₄·7H₂O	27.8	27.8
Na₂·EDTA	37.3	37.3
KI	0.83	0.75
H₃BO₃	6.2	3
CoCl₂·6H₂O	0.025	0.025
MnSO₄·4H₂O	22.3	-
MnSO₄·H₂O	-	10
CuSO₄·5H₂O	0.025	0.025
Na₂MoO₄·2H₂O	0.25	0.25
ZnSO₄·7H₂O	8.6	2
Organic compounds (mg/L)		
Glycine	2	-
myo-Inositol	100	100
Nicotinic acid	0.5	1
Thiamine-HCl	0.1	10
Pyridoxin-HCl	0.5	1

Figure 2.3. Compositions of two commonly used PTC media [71]

Plant growth regulators also have an important effect on SM production. Changing the type, concentration and ratio between auxin/cytokinin has important effects on both cell growth and SM production. 2,4-Dichlorophenoxyacetic acid (2,4-D) is known to be inhibitory for most SMs including anthocyanin production in *Populus* cell cultures; however, there are also reports on 2,4-D that it increases the carotenoid production on *Daucus carota* cultures [82].

Culture conditions such as temperature, light intensity, pH, humidity and oxygen have important effects on the production of SMs. In PTC, generally plants require temperatures in the range of 17-25°C for their growth. Light intensity applied on the culture depends on the SM type whether its production is affected by the light or not. According to the light requirements, cultures are grown in photoperiod or in dark. pH of the medium is generally adjusted between 5-6 initially, however, it fluctuates during cell growth. Ammonia assimilation causes a decrease in the pH whereas nitrate uptake causes an increase [83]. All component of the culture medium and condition has individual effects on production of SMs.

2.3.2. Advanced Techniques for Secondary Metabolite Production in Plant Tissue Culture

2.3.2.1. Elicitation

Elicitors are physical or chemical agents that mimics the biotic or abiotic stress induction in PTC in order to enhance SM production [84]. Application of the elicitors on the growth medium activates stress response which leads to an increase in SM production and this procedure is named as elicitation. There are different types of elicitors including physical, chemical, hormonal, plant-based and fungal elicitors (Figure 2.4.). Its application period, concentration and method depend on the plant species and desired SM to be produced.

Elicitors	Plant Species	Secondary Metabolite	Fold Increase Compared to Control
Pulsed electrical field	<i>Vitis vinifera</i>	Resveratrol	3.6
Jasmonic acid	<i>Calendula officinalis</i>	Oleanolic acid	9.4
Dried cell powder of <i>Phoma</i> spp.	<i>Claothylum inophyllum</i>	Inophyllum	751
Heavy metal (Ag ⁺)	<i>Taxus chinensis</i>	Paclitaxel	4.6

Figure 2.4. Different types of elicitors and their effects on SM production [85–88]

2.3.2.2. Precursor Feeding

Precursor feeding is a technique that is based on the knowledge about plant biosynthetic pathways. It is mainly using an intermediate or an initiator compound in the SM production pathway as a precursor in order to increase the yield by adding the intermediate into the culture. Precursors can be applied to the culture in various stages of growth depending on the target SM. It is an economic strategy to increase the SM production; however, since some plant biosynthetic pathways are still unknown, it becomes a challenge [79]. Increase of nicotine yield on *Nicotiana tabacum* culture by the addition of nornicotine as a precursor can be an example of precursor feeding [83].

2.3.2.3. Biotransformation

Biotransformation is the modification of precursor molecules to organic compounds by plant cells and enzymes. Changing and altering the metabolic pathways by the knowledge of plant biotechnology, biochemistry and genetic engineering is called metabolic engineering. Metabolic engineering analyzes the biotransformation during organic compound production including SMs. These applications can result in the production of non-existing new compounds in PTC. Different model plant cell cultures are used to study biosynthetic pathways and application of metabolic engineering including *Nicotiana tabacum*, *Arabidopsis thaliana* and *Oryza sativa* cell cultures [71,83].

2.3.2.4. Cell Immobilization

Cell immobilization is a technique that is generally used for large-scale production of plant cells which is immobilizing the plant cells into a matrix. Due to slow growth and production of target molecule of plant cells, makes them easily effected by the shear stress especially in large-scale production. Immobilizing the cells protects them from shear-stress which lead to an increase in SM production. There are three main immobilization system types which are physical entrapment system (paraffin, hollow fiber tube), gel matrix entrapment system (alginate, agar, polyacrylamide) and surface immobilization system (polyurethane foam, sisal) [71,76,89].

2.3.2.5. Genetic Engineering

Genetic engineering application on both plant cell cultures and hairy root cultures results in higher SM yield than classical techniques. It has three main steps including isolation and characterization of the gene of interest, reorganizing genetic material and transfer [76]. Genetic manipulations using *Agrobacterium tumefaciens* is one of the most important techniques in gene transfer studies since it is a natural genetic engineer normally infecting dicot plants [90,91]. Other gene transfer techniques include microinjection, electroporation, biolistics, viral transformation and polyethylene glycol (PEG)-mediated gene transfer. Genetic engineering studies was firstly performed in *Lupinus polyphyllus* and *Petunia hybrida* cultures in order to enhance SM production [76].

2.3.2.6. Use of Endophytes

Using endophytic fungi for the improvement of SM production is a relatively new concept. According to the studies on biosynthetic pathways of both plants and mutualist fungi, it has been shown that they have distinct pathways for the production of SMs; however, their mutualistic nature results in higher SM yield. Studies on *Taxus x media* and *Cladosporium cladosporioides* showed that their symbiosis resulted in production of Taxol on both the plant and the fungi. It also resulted in higher SM production [92,93].

2.3.2.7. Molecular Pharming

Molecular pharming is the production of pharmaceutically important SMs through genetically modified plant cell cultures in large-scale. It results in high-purity and high-quality products with a high yield. It can be used for the production of vaccine, enzyme, resistant plant and food production [94].

2.3.2.8. Scale-up Techniques

Bioreactors are advanced systems that important growth parameters such as temperature, pH changes, light intensity, agitation and gas exchange can be controlled. There are different types of bioreactors that can be used in scale-up studies such as stirred-tank, bubble column, air-lift and wave bioreactors. Stirred-tank bioreactors with ring spargers are the most commonly used bioreactor type for plant cells. The main purpose of using bioreactors to

scale-up is to increase biomass production which can be achieved by optimizing growth parameters. However, optimization of each parameter starting with the bioreactor type can be very challenging for plant cells. It is due to the fact that plants have large cell size and tends to create aggregates which results in uneven measurements. When compared with microbial cultures, their doubling time is considerably high and they are easily affected by shear stress. Optimizing each parameter for optimal growth requires time; however, once everything is optimized it is a productive, reproducible and profitable method especially for pharmaceutically important SM production [71,72,95–97].

2.4. *NICOTIANA TABACUM* AND ITS SECONDARY METABOLITES

Nicotiana tabacum L. (tobacco) is a perennial and erect plant that belongs to the *Solanaceae* family. Its name comes from the French ambassador John Nicot who distributed some species throughout Europe from Central and South America in 16th century. *N. tabacum* L. ($2n=4x=48$) is an allotetraploid formed 6 million years ago by the hybridization of *N. sylvestris* ($2n=24$) and *N. tomentosiformis* ($2n=24$) in America and widely used in religious activities by ancient civilizations. Now, it is widely distributed around the world with China being the main producer. *N. tabacum* can grow heights between 1-3 m in well drained warm climate (20-30°C) and produces white-pink flowers (Figure 2.5.). Its growth from seed to next generation seeds takes about 3 months and each plant can produce million seeds. Due to its fast growth, *N. tabacum* has been used as a model plant in PTC and molecular biology studies. However, it is mainly grown for the production of smoking tobacco which is addictive due to high Nicotine content that stimulates central nervous system. Nicotine is one of the most important secondary metabolites found in *N. tabacum*. It is an alkaloid and alkaloids takes up 2 percent of leaf dry weight and 95 percent of it is nicotine that protects plant against herbivores. There are more than 2500 SMs found in *N. tabacum* including major groups like alkaloids, isoprenoids and flavonoids. Capsidiol, rishitin, β -carotene and solanesol are examples to some SMs produced by *N. tabacum* [7,98,99].



Figure 2.5. *Nicotiana tabacum* cv. Xanthi grown in greenhouse conditions

2.4.1. Secondary Metabolites of *Nicotiana tabacum*

Nicotiana tabacum L. (tobacco) is one of the most well studied crops in terms of metabolite production. It has a large genome (4.5 Gb) with 90,000 protein coding genes which results in the production of biologically complex compounds. According to the recent studies, approximately 5700 different chemicals including carbohydrates, alkaloids, pigments, nitrogenous compounds, isoprenoids, sterols and phenolics were found in all tissues of *N. tabacum*. The biosynthetic pathways of primary and secondary metabolites can be seen in Figure 2.6. It can be seen in the figure that different SMs can be produced from the same precursor molecule. *N. tabacum* Being a model organism for PTC studies resulted in extensive research on *N. tabacum* SM production [100–103]. Especially, the alkaloid production by *N. tabacum* is the highest among all *Nicotiana* genus. Two percent of *N. tabacum* dry weight consists of alkaloids with nicotine taking up 95 percent of it. Due to cigarette consumption, nicotine is extensively studied. Other alkaloids produced by *N. tabacum* includes cotinine, nornicotine, myosmine and anabisine. The main purpose of these alkaloids produced by *N. tabacum* are protection against insects/herbivores, detoxification and regulation of growth [7,103].

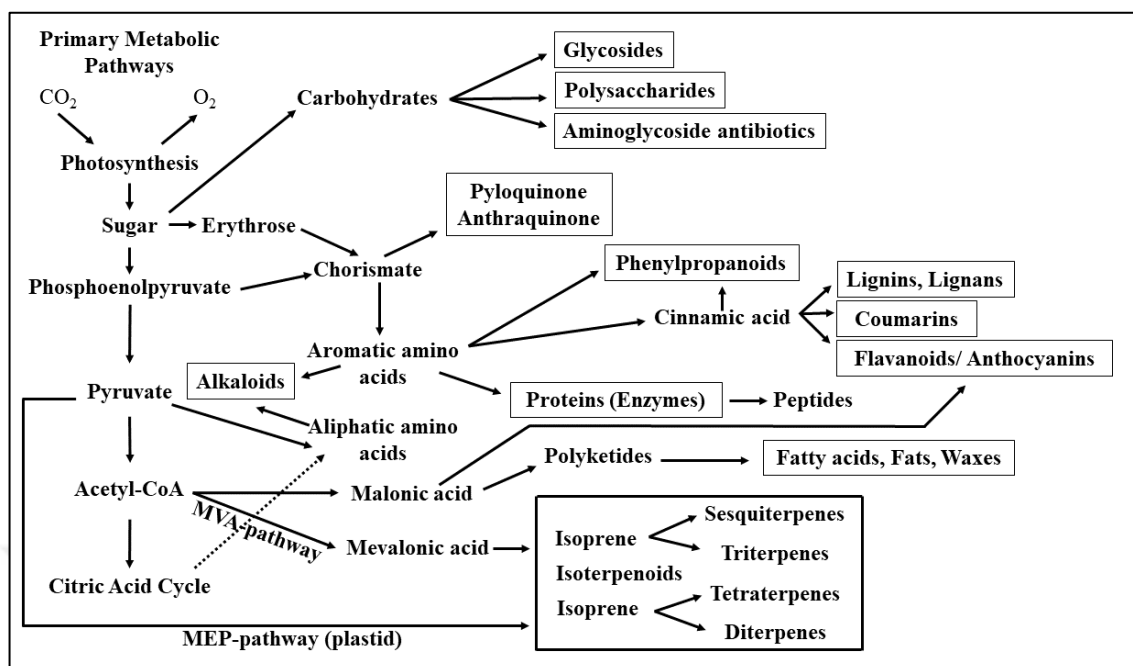


Figure 2.6. Primary and secondary metabolite biosynthesis pathways [103]

Another major SM group synthesized by *N. tabacum* is terpenes (isoprenoids). They are involved in the production of the aroma, protection against microorganisms and also have growth regulatory properties. Diterpenes, tetraterpenes, triterpenes, sterols and sesquiterpenes are some of the major classes of terpenes produced by *N. tabacum*. Carotenes, capsidiol, rishitin, phytuberin, lubimin and solanesol can be examples of *N. tabacum* terpenes. *N. tabacum* terpenes have commercial importance since they include pharmaceutically important compounds [7]. Finally, phenolics produced by *N. tabacum* are responsible for the production of color, protection against insects/herbivores and reactive oxygen species (ROS). There are more than 4000 phenolics produced by *N. tabacum* including anthocyanin, rutin, isoquercetin, karacyanin and astragalin [7,103,104].

2.4.2. Solanesol and Its Biosynthesis

Solanesol ($\text{C}_{45}\text{H}_{74}\text{O}$) is a polyisoprenoid alcohol with nine isoprene units that is found in *Solanaceae* family plants (Figure 2.7.). It was firstly isolated from flue-cured *Nicotiana tabacum*; however, studies on *Solanum lycopersicum* (tomato) and *Solanum tuberosum* (potato) showed that they could also produce solanesol. Chemical synthesis of solanesol is

difficult since it consists of long carbon chains so it is mainly extracted from solanaceous plants. Solanesol has antioxidant, antibacterial, antifungal, anti-inflammatory, anti-cancer and anti-viral properties. It is an important intermediate for the production of pharmaceutically important drugs and supplements such as coenzyme Q10, vitamin K2, vitamin E and N-solanesyl-N,N'-bis(3,4-dimethoxybenzyl) ethylenediamine (SDB) [10,103,105,106].

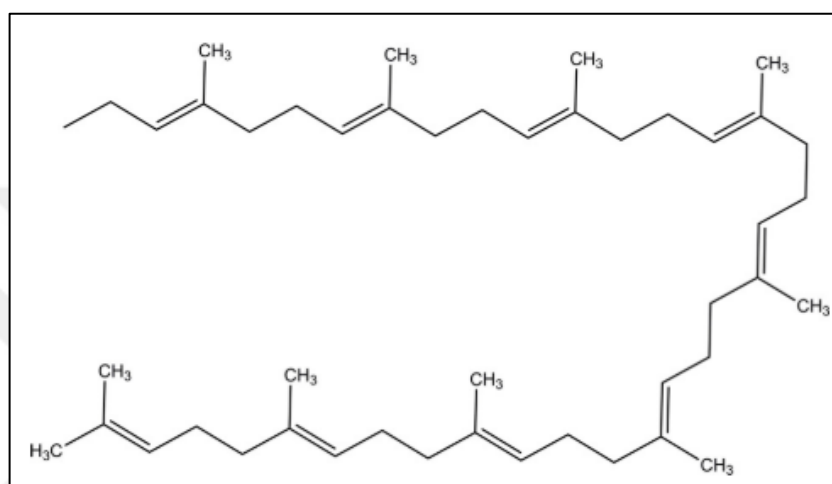


Figure 2.7. Chemical structure of solanesol [107]

Antioxidant activity of solanesol comes from its ability to absorb free radicals due to non-conjugated double bonds in its structure. It was found that solanesol protected liver cells from ethanol induced oxidative injury by upregulating an inducible enzyme that has anti-inflammatory and antioxidant functions. It has also a potential use in inhibition of age spots and ageing due to its UV radiation absorption ability since it inhibits tyrosinase which is a key enzyme in melanin synthesis [108,109]. Anti-inflammatory effects of solanesol was also tested and found to be effective on rats by developing a herbal gel (consisting of solanesol from tobacco scrap and gelling agents) against hind paw oedema induced by carrageenan [110]. Solanesol also has antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa* and *Mycobacterium phlei* [105].

Medicinal value of solanesol comes from its use as an intermediate for the synthesis of ubiquinone drugs such as coenzyme Q-10, vitamin K2 and SDB. Coenzyme Q-10 is a lipid soluble quinone that plays an essential role in mitochondrial respiratory chain by participating oxidative phosphorylation and ATP synthesis. It is an activator of cellular

respiration and metabolism. Coenzyme Q-10 can be used for the treatments of diseases such as neurodegenerative diseases, hypertension and cardiovascular diseases since it has high antioxidant activity, enhancement of immunity and strengthening of cardiac power and brain function [111]. Vitamin K2 is a lipid soluble vitamin that has effects on prevention and treatment of osteoporosis, promoting blood clotting and inhibition of vascular calcification. SDB has been found to reverse the drug resistance of paclitaxel resistant cell lines and it can be used as anti-cancer drug. Solanesol on its own can reduce the toxicity of traditional cancer drugs and enhance the tumor suppression effects [105,112,113].

Solanesol biosynthesis starts with common five carbon isoprene unit precursors synthesized in both MVA and MEP pathways called isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Figure 2.8.). Synthesis in MVA pathway takes place in cytoplasm and Acetyl-CoA is used as the building block. Meanwhile, synthesis in MEP pathway takes place in plastids and it uses pyruvate and glyceraldehyde-3-phosphate (GAP) as building blocks. IPP is shuttled between the cytoplasm and plastids during solanesol synthesis so MVA and MEP pathways work together, however, solanesol molecule is synthesized in plastids through MEP pathway. In MEP pathway, 1-deoxy-D-xylulose-5-phosphate (DXP) is synthesized from pyruvate and GAP via 1-deoxy-D-xylulose-5-phosphate synthase (DXS) enzyme which is the starting point for the formation of five carbon units. With 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) enzyme, molecular rearrangement and reduction on DXP results in the formation of 2-C-methyl-D-erythritol-4-phosphate (MEP). Through catalytic reactions, MEP forms IPP and DMAPP. Isopentenyl diphosphate isomerase (IPI) enzyme catalyzes the interconversion between DMAPP (C₅) and IPP(C₅).

Geranyl diphosphate (GPS), farnesyl diphosphate (FPP) and geranyl geranyl diphosphate (GGPP) are respectively synthesized from IPP and DMAPP. Solanesyl diphosphate (SPP) is synthesized from IPP, DMAPP, GPP, FPP and GGPP via solanesyl diphosphate synthase (SPS) enzyme. Finally, conversion of SPP pyrophosphate groups to hydroxyl groups leads to the synthesis of solanesol [10,103,114].

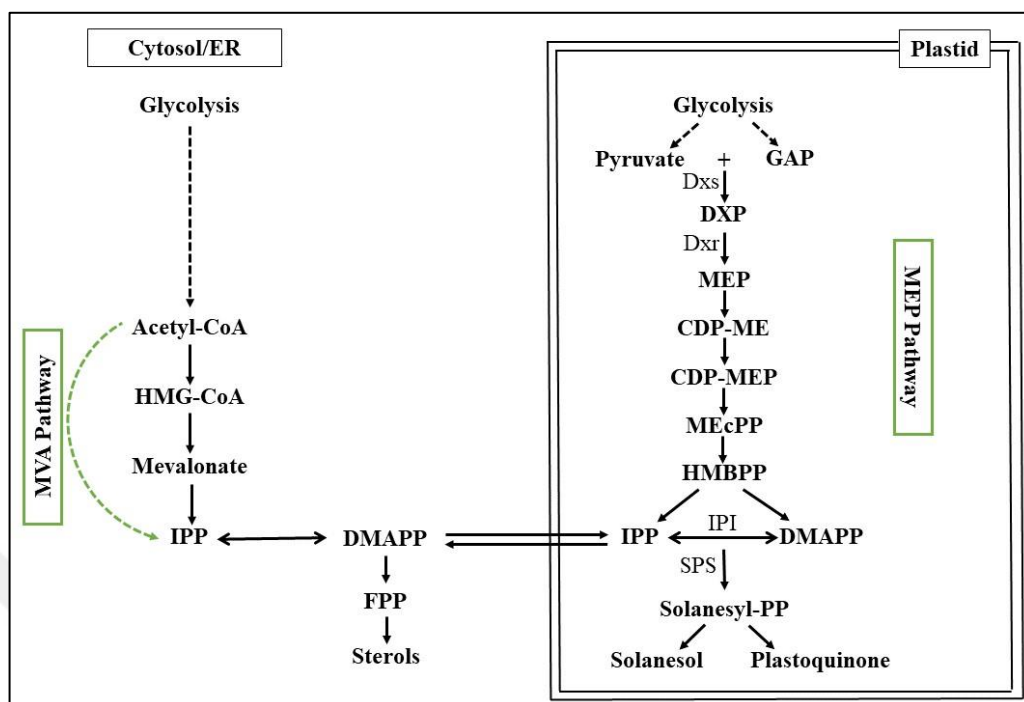


Figure 2.8. Solanesol biosynthesis in plants [103,107]

Solanesol is present in *Solanaceae* family plants such as *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Solanum melongena* (eggplant) and *Capsicum annum* (pepper); however, *N. tabacum* (tobacco) has the highest solanesol content among all solanaceous plants with highest concentration being on the leaves (0.04-3.6 percent of DW). Solanesol concentration have major differences in different part of the tobacco plant with 0.037 percent in peduncle, 0.0037 percent in stalk and 0.0013 percent in root that can be seen in Table 2.1. with other plant species. The concentration of the solanesol can be influenced by genetic variation, stalk position, growth period, developmental stage, number of leaves and curing process. Solanesol is found in the plants in two states which are free and ester bounded with oleic, myristic, linoleic and/or palmitic acid [9,114,115]. Approximately 68 percent of solanesol in tobacco leaves are found in bound state; however, this amount changes according to the growth stage of the plant. Solanesol content of the plants is determined by both environmental and genetic factors; however, there is limited information about what is effecting its accumulation levels [9].

Table 2.1. Solanesol content differences in plant species and tissues

Plant species	Tissue	Solanesol content (% dry weight)	References
<i>Nicotiana tabacum</i>	Leaf	1.78-3.6	[9]
<i>Nicotiana tabacum</i>	Leaf	0.64-0.57	[116]
	Stalk	0.19-0.06	
	Flower	0.03	
<i>Nicotiana tabacum</i>	Leaf	0.45	[117]
	Peduncle	0.037	
	Stalk	0.0037	
	Root	0.0013	
<i>Solanum tuberosum</i>	Leaf	0.4	[116]
<i>Solanum tuberosum</i>	Leaf	0.04	
<i>Capsicum annuum</i>	Leaf	0.35	
<i>Solanum lycopersicum</i>	Leaf	0.18-1.0	[118]

Solanesol is normally white waxy solid in its pure form; however, its color changes to yellow with the increasing impurities. It is insoluble in water but soluble in organic solvents. Its UV absorption band is between 195-220 nm. Solanesol is hard to extract, purify and analyze. Different analytical techniques are used in order to analyze metabolites in biological samples. However, biological samples need to be analyzed after pretreatments such as dilution, filtration, precipitation and extraction to obtain target metabolites with minimized matrix effect. Most commonly used techniques are liquid-liquid extraction (LLE), solid-liquid extraction (SPE) and precipitation [119]. In extraction process of solanesol, firstly bound solanesol needs to be released (requires a saponification reaction) and the total solanesol is extracted afterwards. Saponification is generally achieved with either 10 percent ethanolic potassium hydroxide, NaOH in ethanol or 0.02 M NaOH. The optimal

saponification period should not exceed 4 hours since solanesol levels decreases after that period due to hydrolysis process. During this process heat-reflux extraction method is abundantly used. Generally in extraction process, solanesol like substances and phenolics are extracted with the solanesol such as chlorogenic acid, rutin, caffeic acid, solanesene and farnesene [9,115]. There are also different methods for the extraction of solanesol such as ammonia leaching pretreatment assisted extraction, dynamic saponification extraction, ultrasonic assisted extraction, supercritical fluid extraction, bioenzymatic extraction, molecular distillation extraction and molecular imprinted polymers extraction. Each method can be used on their own and with others to enhance the purity [105]. In order to extract solanesol, combination of these methods can be used.

For the quantification of solanesol, chromatography methods like high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), thin layer chromatography (TLC) and gas chromatography (GC) are the most widely used methods. Reversed phase high performance liquid chromatography (RP-HPLC) with UV detector is the most common method. It utilizes methanol, acetonitrile, hexane, isopropanol and their combinations in different ratios as mobile phase. RP-HPLC with C₁₈ column and UV detector is the commonly used method for solanesol analysis [105,118,120]

Solanesol is commercially produced from tobacco waste which consists of stems, midrib, low grade leaves and root that are not used in the production of smoking tobacco. Since the waste contains mostly stems, midrib and roots, solanesol levels changes. Also curing type and period changes for the production of different types of smoking tobacco which results in the changing levels of solanesol. Combination of these results in obtaining inconsistent amounts of solanesol with low purity. There are two types of solanesol in the market with different purities. Brown cream solanesol has the purity around 16 percent whereas faint yellow solanesol has purity around 75 percent. However, there are no defined regulations or standards on the solanesol manufacturing. According to the investigations on different manufacturers, mainly in China and Japan, every product had 10 percent lower solanesol concentration than stated. The demand for high purity solanesol expected to increase more than 66000 tons by 2022 from being 4000 tons in early 2000s. With increasing demand from the pharmaceutical industry, new methods needs to be found for the production of solanesol [118,120,121].

2.5. AIM OF THE STUDY

The objective of this study is to extract solanesol from *Nicotiana tabacum* cv. Xanthi cell suspension culture and scale-up the process of cell suspension cultures.

In order to achieve this goal, the following studies have been conducted; 1) Maintenance of a stable *Nicotiana tabacum* cv. Xanthi cell suspension culture, 2) Analyzing the growth parameters of cell suspension culture, 3) Extraction of solanesol from *N. tabacum* cv. Xanthi cell suspension culture, 4) Analyzing the solanesol accumulation in cell suspension culture through HPLC analysis, and 5) Scale-up *N. tabacum* cv. Xanthi cell suspension culture for large scale production via stirred-tank bioreactor.

To our knowledge, this is the first study that focuses on the extraction of solanesol from plant cell suspension culture instead of extracting from whole plant or industrial waste.



3. MATERIALS

3.1. CHEMICALS

Murashige & Skoog medium including vitamins (Duchefa Biochemie, M0222.0050), Sucrose crystallized (Duchefa Biochemie, S0809.1000), Casein hydrolysate (Duchefa Biochemie, C1301.0250), Agar plant tissue culture (Pyhtotechlab, A296), 6-Benzylaminopurine (Caisson Laboratories, B001-25GM), 1-Naphtaleneacetic acid (Sigma, NO640-25G), Charcoal steam activated (Duchefa Biochemie, C1302.1000), Ethanol (UmayLab), Ethanol HPLC grade (J.T. Baker, 10538071), Acetone (UmayLab), D-Fructose (BioChemica, A3688), D-Glucose (Sigma, A1422), D-Sucrose (Fisher Scientific, S5-500), Acetonitrile HPLC grade (J.T. Baker, 02-002-180), 2-Propanol HPLC grade (Sigma-Aldrich, 34863), Hexane (Sigma-Aldrich, 296090), Solanesol from tobacco (Sigma, S8754).

3.2. GLASSWARE & CONSUMABLES

Micropipettes 0.1-10 μ l, 20-200 μ l, 100-1000 μ l, Micropipette tips 10 μ l, 100 μ l, 1000 μ l, Erlenmeyer flasks 50 ml, 100 ml, 250 ml, 500 ml, Conical bottom clear centrifuge tubes 15 ml and 50 ml, Petri dishes 90 mm, Square magenta boxes, Eppendorf tubes 2 ml, Weighing dishes, Graduated cylinders 50 ml, 100 ml, 500 ml, 1 L, Borasilicate glass bottles 100 ml, 250 ml, 500 ml, 1 L, 2 L, Glass beakers 100 ml and 2 L, Teflon callus spoon, Forceps, Blade holders, Scalpel blades no:10 and no: 11, Gooch funnels with sintered glass discs 125 ml and 500 ml with porosity 2, 3, 4, Glass funnel, Funnel holder, Separation funnel 250 ml, Filter glass with side arm 1L and 2L, Protective collar for filter glass, Pear shaped glass flask 100 ml, Evaporation clips, Filter paper, Parafilm, Cling film, Aluminum foil, Tubing clamps, Tubing silicones, Porcelain mortar and pestles, Tube racks, Wash bottles, Spray bottles, Pasteur pipettes, Serological pipettes, Suction bulb, Glass beads, Laboratory brushes, Test indicator for steam sterilization, Gloves, Magnetic stirring bars, Sterile syringes 2 ml, 10 ml, 50 ml, RC membrane filters 0.45 μ m, 0.22 μ m, Glass chromatography vials 2 ml, Chromatography inserts, Microscope slides and cover glasses, HPLC column (Bio-Rad Aminex HPX-87H (300 x 7.8 mm, 9 μ m particle size)), HPLC column Agilent Zorbax Eclipse XDB-C18 reversed phase column (4.6 x 150 mm, 5 μ m particle size)).

3.3. EQUIPMENT

Class II biosafety cabinet (Esco, AC2-4E8), Orbital Shaker (Daihan Scientific, Thermostable IS-10RL), Water purification system (Merck, Milipore Milli-Q Gradient A10), Plant growth room (Grotech, GR94), Incubator/ Drying oven (Wisd, Thermostable IR-150), Heat sterilizer (Swiss made, Steri 350), pH meter (Mettler Toledo, Seven compact), Autoclave (Wisd, MeXterile 60), Analytical scale (Shimadzu, Uni Block TW423L), Hot plate-magnetic stirrer (IKA Labortechnik), Vortex (Wisd, WiseMix VM-10), Ultrasonic bath (Fisherbrand, FB15053), Diaphragm vacuum pump (KNF Neuberger, N022A_.18), Water bath (Grant, SUB Aqua 26 Plus), Rotary evaporator (Heidolph, Laborata 4000) High performance liquid chromatography (Shimadzu, UFLC-XR), Bioreactor (Infors HT, Minifors 2), Peristaltic pump (Watson Marlow, 101U/R).

3.4. PLANT MATERIAL

Nicotiana tabacum cv. Xanthi seeds, cell lines and dry tobacco leaves that were previously produced by Yeditepe University Department of Genetics and Bioengineering were used throughout this study.

4. METHODS

4.1. MEDIA COMPOSITION

Media compositions that were used in this study are given in Table 4.1. All media compositions were optimized in a previous study [122].

Table 4.1. *Nicotiana tabacum* cv. Xanthi media compositions used in this study.

	MS (including vitamins) (g/L)	Sucrose (g/L)	Casein hydrolysate (mg/L)	NAA (mg/L)	BAP (mg/L)	Agar (g/L)	Charcoal (g/L)
Germination media (NtGM2)	4.405	30	-	-	-	6.8	1
Micropropagation media (MS1BACH)	4.405	30	250	-	1.0	6.8	-
Callus induction media (NtCIM3)	4.405	30	250	3.0	0.2	6.8	-
Suspension media (NtCIM5)	4.405	30	250	2.5	0.2	-	-

4.2. CALLUS INDUCTION AND CELL LINE MAINTENANCE OF *NICOTIANA TABACUM* CV. XANTHI

Nicotiana tabacum cv. Xanthi cell lines were maintained and used throughout this study. They were maintained in plant growth rooms (25°C, 65 percent humidity, photoperiod 16h light/8h dark) and subcultured every 3-4 weeks into fresh NtCIM3 medium.

Meanwhile, new calli were induced from seeds that were produced in greenhouse conditions and stored at +4°C. Callus induction started with the preparation of NtGM2 media and sterilization of *N. tabacum* cv. Xanthi seeds. NtGM2 media was sterilized with autoclave and poured into magenta boxes. For sterilization of the seeds, they were placed inside three

layers of filter paper and closed before sterilization inside a biosafety cabinet. Seeds were submerged into 70 percent Ethanol for 8 minutes then washed with autoclaved double distilled water (ddH₂O) two times for 1 minute each. After that seeds were placed in 2 percent NaOCl for 5 minutes and washed with ddH₂O six times for 1 minute each. Finally, seeds were rinsed with ddH₂O for 30 minutes and transferred into NtGM2 medium by placing 5 seeds to each magenta box. Magenta boxes containing the sterilized seeds were placed in +4°C for 18 hours and transferred to plant growth room.

One week after sterilization germinated seeds were calculated. Two weeks after germination, explants were taken by cutting 2-5 mm from the leaves. Leaf explants were placed in NtCIM3 medium for callus induction. Petri dishes containing the explants were placed in an incubator at 25°C, dark. Callus formation around the explants were observed after 7 days. Calli were transferred into fresh NtCIM3 medium and placed in plant growth room. Newly grown calli were subcultured every 3-4 weeks.

Plantlets taken from the newly germinated seeds were also placed in MS1BACH medium for micropropagation and maintained in plant growth room by subculturing every 5-6 weeks.

4.3. MAINTENANCE OF STABLE *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURE

Nicotiana tabacum cv. Xanthi cell suspension culture was initiated from callus cell lines. Calli were placed in a sterilized petri dish and cut into approximately 1 mm pieces by a blade. 250 ml Erlenmeyer flasks (containing 100 ml NtCIM5 medium) were sterilized and approximately 5 g of calli were transferred into each flask. Flasks were placed in an orbital shaker under light at 25°C, 110 rpm and subcultured after 7 days. A vacuum filtration system and laboratory scale were used for subculturing the suspensions. Flasks were poured into the vacuum filtration system and the cells were weighed by a laboratory scale before subculturing into fresh medium. *N. tabacum* cv. Xanthi cell suspension cultures became homogenous by discarding cell aggregates in every subculture. After 6-8 subcultures suspensions became homogenous and they were maintained by subculturing weekly throughout the study. Growth rate of the cell suspension culture were calculated for 21 subcultures by subtracting final cell mass (g) from initial cell mass (g) and dividing it into culture period (days) times volume (L) [66,123].

4.4. DETERMINATION OF CELL VIABILITY OF *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURE

Viability of the cells were confirmed after obtaining a homogenous cell suspension culture by viability test with fluorescein diacetate (FDA). Suspension cells were observed under light microscope before starting the viability test. After that, FDA stock was prepared by dissolving 5 mg of FDA in 1 ml Acetone. 5 ml sample was taken from cell suspension culture into a 15 ml falcon tube. 950 μ l fresh NtCIM5 medium and 1 ml of cell suspension were mixed in a 2 ml Eppendorf tube. 50 μ l FDA was added to the Eppendorf tube and incubated in dark for 5 minutes. After incubation, samples were placed in a microscope slide and observed under a fluorescent microscope. Living cells were observed as green due to the illumination of fluorescent molecule.

4.5. CONSTRUCTION OF GROWTH CURVE FOR *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURE

Growth parameters were analyzed for the cells at their eight subculture for 15 days. Measurements were taken in every other day. 100 ml Erlenmeyer flasks containing 20 ml of NtCIM5 medium were used to initiate growth curve analysis. 1 g of *N. tabacum* cv. Xanthi cells from the suspension were weighed during weekly subculturing and placed into each flask (3 biological replicates for each day with total of 24 samples). Flasks were placed in orbital shaker under light at 25°C, 110 rpm. Fresh weight (FW), dry weight (DW), pH and sugar consumption were measured every other day for 15 days. Fresh weight was measured by a laboratory scale after filtering the cells and washing with autoclaved distilled water for two times. The filtrate from each sample was collected at the same time for pH and sugar consumption analysis. 2 ml from each sample were stored in -20°C until HPLC analysis of sugars. pH of each sample were measured via pH-meter. Dry weight was measured by a laboratory scale after drying these cells in a filter paper at 85°C for two days.

4.6. EXTRACTION OF SOLANESOL FROM *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURE

In every subculture, remaining suspension cells were collected and stored in -20°C in 50 ml falcon tubes for solanesol extraction. Cells were washed 3 times with distilled water (dH₂O) and dried inside a filter paper for 12 hours at 65°C. After drying process of the cells, cells and dried tobacco leaves were grounded by mortar and pestle. 2 g of each grounded sample were placed in 100 ml Erlenmeyer flasks (3 replicates of both cells and dry tobacco leaves). 20 ml of EtOH and 0.4 g KOH were added to the flasks. Sealed flasks were placed in a water-bath at 60°C for 4 hours to ensure the hydrolysis of the bound solanesol. After incubation, flasks were cooled to room temperature and filter through a filter paper via a glass funnel. The filtrates were diluted to 35 ml volume with dH₂O and cell residues were placed in 50 ml falcon tubes for further extraction. Filtrates were extracted with n-hexane for 4 times (volume of n-hexane: 20, 20, 15 and 15) in a separation funnel by vigorously shaking (liquid-liquid extraction). Cell residues were ultrasonically extracted 4 times with 20 ml n-hexane for 20 minutes (solid-liquid extraction). Then, residues were filtered through a filter paper. Filtrates coming from liquid-liquid and solid-liquid extractions were combined and evaporated in a rotary evaporator at 40°C. Residues after evaporation were dissolved in 1 ml Acetonitrile and stored in -20°C until HPLC analysis [124].

4.7. HPLC ANALYSIS FOR THE QUANTIFICATION OF SOLANESOL

HPLC analysis of the sugars were carried out using Bio-rad Aminex HPX-87H (300 x 7.8 mm, 9 µm particle size) column with column temperature of 40°C, refractive index detector, 5 mM H₂SO₄ mobile phase, 0.6 ml/min flow, 20 µl injection volume and 20 minutes of run time. Samples were filtered through 0.22 µm RC membrane filters before injection. Sucrose, glucose and fructose standards were prepared by dissolving each of them in double distilled water [125].

HPLC analysis of solanesol extraction samples were carried out using Agilent Zorbax Eclipse XDB-C18 reversed phase column (4.6 x 150 mm, 5 µm particle size) with column temperature of 25°C, UV detector (210 nm), 20 µl injection volume, Acetonitrile: Isopropanol (80:20) mobile phase, 0.7 ml/min flow and 35 minutes of run time. Samples

were filtered through 0.22 μm RC membrane filters before injection. Solanesol standards were prepared by dissolving HPLC grade solanesol standard in Acetonitrile [118].

4.8. PARAMETER OPTIMIZATION FOR *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURE SCALE-UP

In order to scale-up *Nicotiana tabacum* cv. Xanthi cell suspension cultures Infors HT Minifors 2 bioreactor was used. 6 L glass bioreactor with a working volume between 1.1 L-4L was used in this study. Bioreactor had the dimensions of 455 mm x 375 mm x 740 mm (W x D x H) with two Rushton turbine impellers (6 blades- 4 cm in diameter). It was also equipped with pH and pO₂ sensors. 3 L of NtCIM5 medium was prepared and autoclave sterilized (121°C, 15 min) with the bioreactor before each cultivation. pH probe was calibrated before sterilization and dissolved oxygen probe was calibrated prior to inoculation to 100 percent with the use of the sparger. Two reading lamps were used as light source for the cells. Cells were weighed and suspended in minimum amount of fresh NtCIM5 medium before inoculation. Inoculation was carried out using a glass funnel with heat sterilization. 5 different trials were carried out for parameter optimization to scale-up. Parameter changes in each trial can be seen in Table 4.2. In each trial, 15 ml of culture sample was taken with a peristaltic pump under sterile conditions. Sugar consumption analysis was carried out with HPLC analysis of each sample taken from bioreactor [126].

Table 4.2. Parameter changes in *N. tabacum* cv. Xanthi scale-up studies

Number of trials	Temperature (°C)	Stirrer (rpm)	pH control	pO ₂ (%)	Air flow (L.min ⁻¹)	Inoculant size (g)	Batch time (days)
1	25	150	5.9 (1 day after starting the batch)	>30	1.29	80	2
2	25	150	-	>30	1.29	55	7
3	25	75	-	>30	1.29	60	3
4	25	75	6.0	>30	0.6	60	7
5	25	75	6.0	>30	0.6	60	6

4.9. STATISTICAL ANALYSIS

Results of this study were shown as mean $\pm$ standard deviation throughout. Student's t-test was applied to the data acquired and $p \leq 0.05$ was accepted as statistically significant. Minimum of three replicates were used throughout the study.



5. RESULTS AND DISCUSSION

5.1. CALLUS INDUCTION AND MAINTENANCE OF CELL LINES

Maintaining and preserving plant cell lines in laboratory conditions is important for the consistency of the experiment. In this case it was important for consistent SM production. In order to achieve consistency, *Nicotiana tabacum* cv. Xanthi leaf cell lines were maintained throughout the study in NtCIM3 media by regular subculturing as it can be seen in Figure 5.1. NtCIM3 media consists of MS medium (including vitamins), sucrose, casein and PGRs. MS medium was the macro and micro nutrient source, sucrose was the carbon source and casein was the nitrogen source. In addition to those PGRs were used to induce callus formation and maintenance.

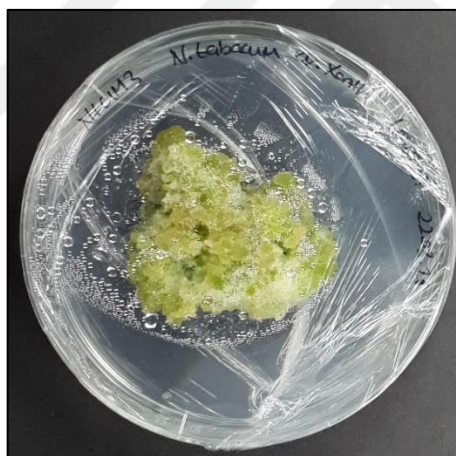


Figure 5.1. *Nicotiana tabacum* cv. Xanthi leaf cell lines maintained in NtCIM3 media and used throughout the study

While maintaining *N. tabacum* cell lines new calli and micropropagations were induced from the seeds as a backup. Both of them started with the seed germination in NtGM2 media which has MS, sucrose, agar and charcoal. Charcoal creates soil like structure when mixed into the media and stimulates the germination while removing phenolic compounds [127]. In this study, seeds germinated in 7 days and the germination rate was calculated as 90.32 percent. Germinated *N. tabacum* was used to induce callus and micropropagation. Germinated *N. tabacum* in NtGM2 medium can be seen in Figure 5.2.

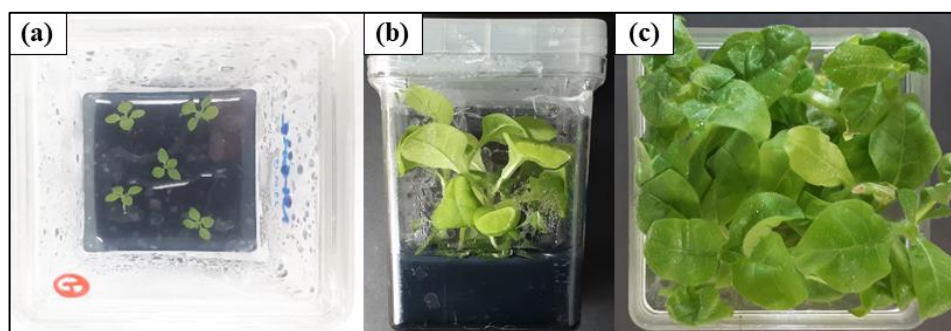


Figure 5.2. *Nicotiana tabacum* cv. Xanthi in NtGM2 media (a) after seed germination, (b) before callus induction, (c) before starting micropropagation

Explants taken from the leaves were used to induce callus formation. They were placed in NtCIM3 media and kept in dark. Keeping the explants in a dark and nutrient rich environment forced them to use the media around them instead of photosynthesis. This resulted in the consumption of the PGRs inside the media which lead to callus formation. In this study, calli formed around the leaf explants in 7 days and callus induction rate was 100 percent. After callus formation, calli were transferred to plant growth room which is in photoperiod. Keeping the calli in photoperiod throughout the study was important for calli to have some capacity of photosynthesis while maintaining its callus form with increased biomass production [128]. *N. tabacum* callus induction and maintenance in this study can be seen in Figure 5.3.

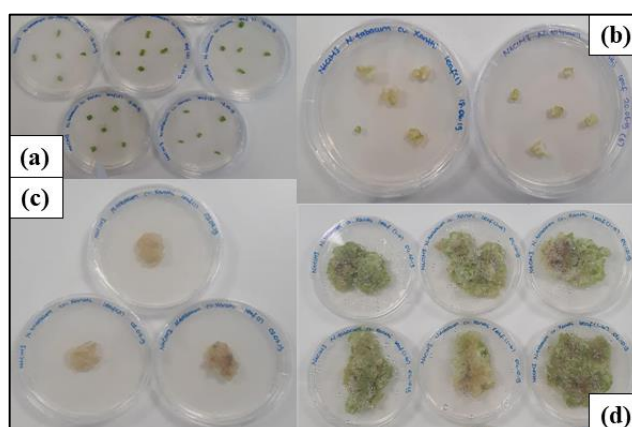


Figure 5.3. *Nicotiana tabacum* cv. Xanthi callus induction on NtCIM3 media (a) explants taken from the leaves for induction, (b) start of callus formation, (c) calli before transferring to plant growth room, and (d) calli in plant growth room

Plants taken from the *N. tabacum* cv. Xanthi were placed in MS1BACH media which has only BAP (cytokinin) as PGR. Using only BAP increased the adventitious bud formation. In this study, micropropagations were regularly subcultured like calli and maintained throughout the study. The growth between subcultures can be seen in Figure 5.4.

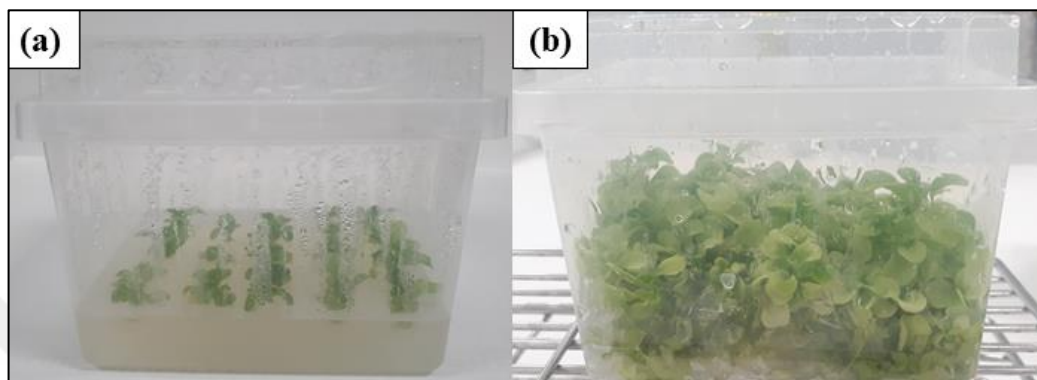


Figure 5.4. *Nicotiana tabacum* cv. Xanthi micropropagations in MS1BACH media (a) one week after subculture, and (b) 4 weeks after subculture

5.2. MAINTENANCE OF *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURES

Nicotiana tabacum cv. Xanthi leaf cell lines were used to initiate cell suspension cultures to produce secondary metabolites. Calli were cut into small pieces in order to break down its rigid structure to obtain homogenous cell suspension culture. Achieving a homogenous cell suspension culture is important. The subculturing process to achieve this can be seen in Figure 5.5 and 5.6. When compared with other plants, *N. tabacum* have higher potential to form clusters so they need be cut down or removed from the suspension culture because cells inside the clusters cannot absorb nutrients and starts to die. Intersect of a cell cluster can be seen in Figure 5.7. In this study, *N. tabacum* cv. Xanthi cell suspension cultures were initiated with approximately 5 g of cell in 100 ml NtCIM5 media. NtCIM5 media have the macro and micronutrients coming from MS, carbon source from the sucrose, nitrogen source from casein and PGRs. Plant cells choose to use sucrose as their primary carbon source which is initially break down into glucose and fructose by the heat of the autoclave and further hydrolyzed by the invertase enzyme secreted from the cells [129]. *N. tabacum* requires high amount of nitrogen for growth so the media was supplemented with casein.

Induced *N. tabacum* cell suspension cultures (in 250 ml Erlenmeyer flasks) were placed in an orbital shaker under light at 25°C, 110 rpm. Shaking the flasks at the right rpm was important to achieve a homogenous cell suspension culture with minimal shear stress so 110 rpm was found to be optimum for *N. tabacum* cell suspension cultures in this study. Cell suspension cultures were weekly subcultured into fresh NtCIM5 medium by vacuum filtration system. In every subculture, cell mass was weighed and recorded for the 21 subcultures. State of the *N. tabacum* cell suspension cultures between subcultures and the vacuum filtration system can be seen in Figure 5.5. and Figure 5.6.



Figure 5.5. *Nicotiana tabacum* cv. Xanthi cell suspension cultures in NtCIM5 media at 18th subculture (a) after subculture, and (b) before subculture



Figure 5.6. Cell suspension culture subculturing setup inside the biosafety cabinet

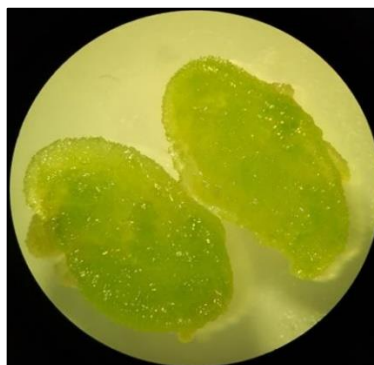


Figure 5.7. Stereo microscope image of *N. tabacum* cv. Xanthi cluster cell intersect with 32x magnification

In this study, cell biomass in every subculture were measured for 21 subcultures and growth rates were calculated by subtracting final cell mass (g) from initial cell mass (g) and dividing it into culture period (days) times volume (L). According to this calculation, in first subculture *N. tabacum* cv. Xanthi cell suspension cultures had 6.9 ± 2.09 gFW/L.D growth rate and it was around this rate until 8th subculture. From that point on growth rate increased even up to 27.48 ± 3.48 gFW/L.D. When first 7 subcultures are compared with the remaining of the study via t-test, every subculture from 8th to 21st had significant difference with $p < 0.05$ except 13th subculture. This proved that *N. tabacum* cell suspensions were adjusted to the suspension conditions and became homogenous from 6th to 8th subculture and maintenance was achieved between 8th subculture to 21st subculture. During this period average growth rate was 20.588 ± 5.9 gFW/L.D. The growth rate changes through 21 subcultures can be seen in Figure 5.8.

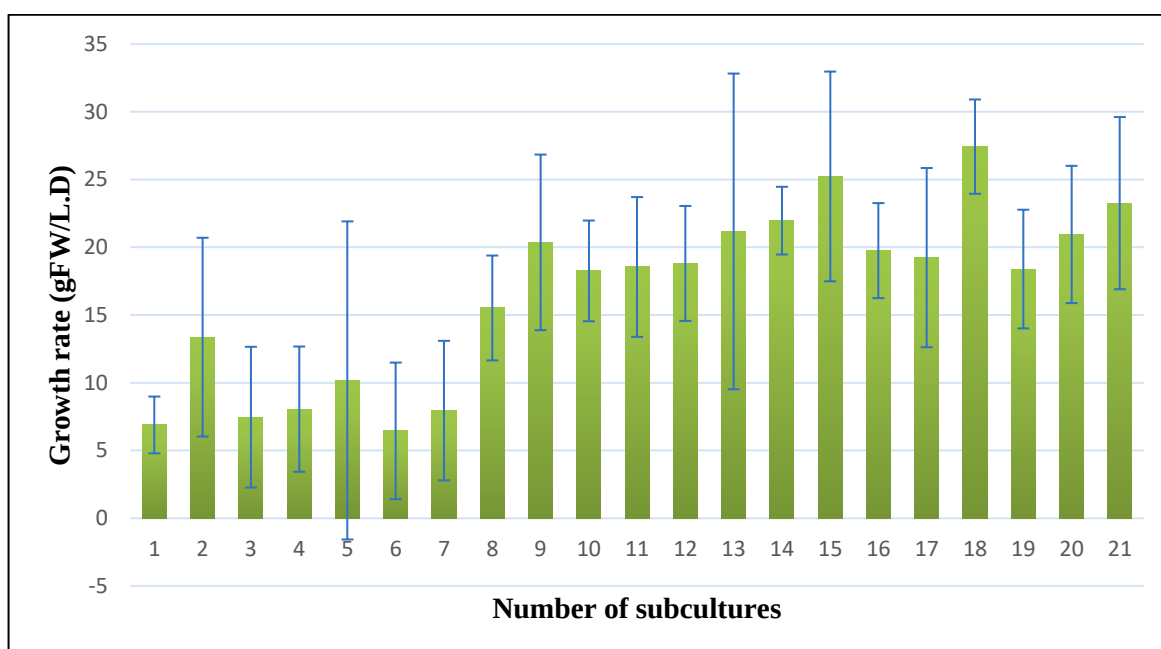


Figure 5.8. *Nicotiana tabacum* cv. Xanthi cell suspension cultures growth rate changes throughout 21 subcultures of maintenance

After achieving a homogenous *N. tabacum* cv. Xanthi cell suspension culture in 8th subculture, cell viability was also confirmed using FDA test. FDA is an esterase substrate that can be absorbed by living cells. When entered to cell, hydrolysis by membrane bound esterases creates fluorescence [130]. Living cells can be observed with bright green color through a florescent microscope with FITC filter. In Figure 5.9., *N. tabacum* cells from suspension were observed through light microscope with 4x and 10x magnification. They had elongated structures and clusters. In Figure 5.10., living cells were observed with bright green color by fluorescent microscope. With growth rates and viability test, it was confirmed that *N. tabacum* cells in suspension cultures were mostly consisted of living cells.

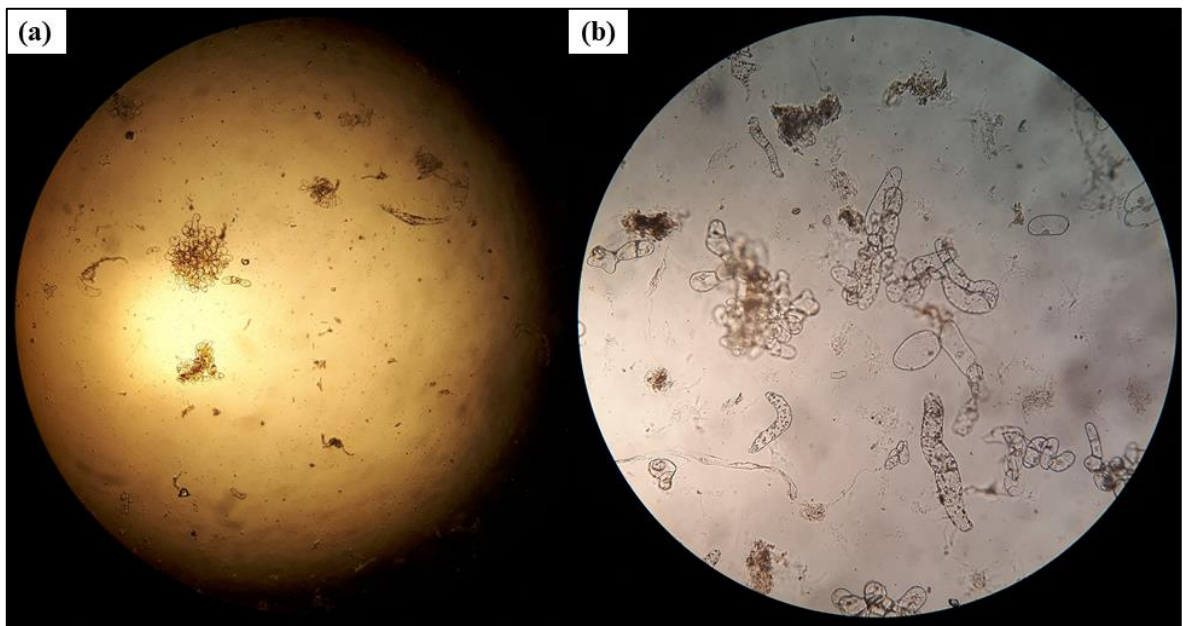


Figure 5.9. *Nicotiana tabacum* cv. Xanthi cell suspension light microscope images (a) 4x magnification, and (b) 10x magnification

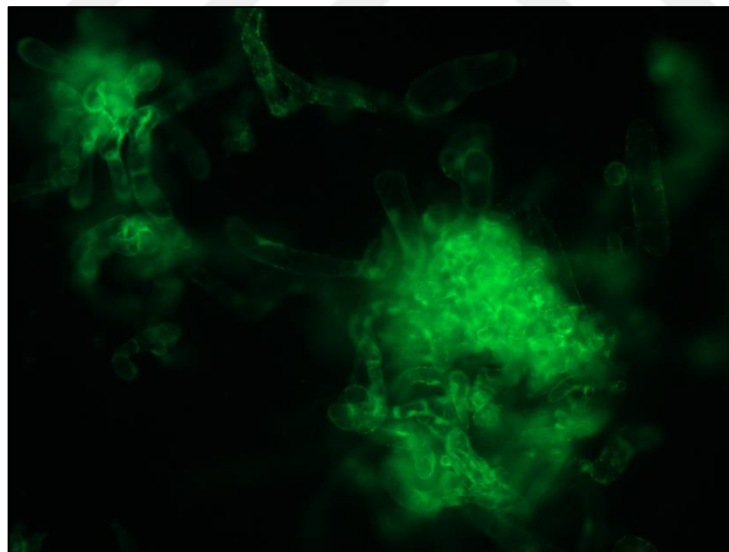


Figure 5.10. Viability test applied to *N. tabacum* cv. Xanthi cell suspension cultures showing living cells with bright green color under fluorescent microscope with FITC filter

5.3. GROWTH CURVE ANALYSIS OF *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURES

Growth curve analysis was conducted on *N. tabacum* cv. Xanthi cell suspension cultures in order to understand the growth dynamics in a 15 days period. Growth phases, optimal pH conditions and biomass changes were determined. Knowing these parameters are important for both SM production and scale-up studies. In this study, growth curve analysis was initiated in 100 ml Erlenmeyer flasks with 20 ml NtCIM5 media using approximately 1g of cell. Three biological replicates were prepared for each day that measurements were taken which are days 1, 3, 5, 7, 9, 11, 13 and 15. Cell suspension cultures used in this study can be seen in Table 5.1. Fresh weight (FW), dry weight (DW) , pH and sugar consumption were examined for 15 days by taking samples every other day. Their final state and all the measurements can be seen in Appendix A.

Table 5.1. *Nicotiana tabacum* cv. Xanthi cell suspension cultures used for the construction of growth curve with three biological replicates

	Replicate 1 (R1)	Replicate 2 (R2)	Replicate 3 (R3)		Replicate 1 (R1)	Replicate 2 (R2)	Replicate 3 (R3)
Day 1				Day 9			
Day 3				Day 11			
Day 5				Day 13			
Day 7				Day 15			

When FW changes were examined for *N. tabacum* cell suspension cultures, starting point was 50 ± 0.85 g/L (1 g cell in 20 ml NtCIM5). However, in the measurements FW decreases drastically in the first day which can be seen in Figure 5.11. This probably happened due to liquid content while weighing the cells to initiate growth curve. In order to minimize the stress while subculturing into a fresh media through vacuum filtration, small amount of media is left around the cells. However, while weighing the cells for FW measurements cells are completely rinsed and dried from the media to get accurate results since they are not going to be used again for subculturing. It can be seen that FW of the cells increased continuously until day 15 except day 11. Since each flask has its own environment one of the flasks containing replicate of day 11 were not in good condition so its measurements affected the average and increased the standard deviation. On day 15, average of 164 ± 4.29 g/L *N. tabacum* cell suspension FW was obtained which is 3.15 times the initial amount (approximately 50 g/L).

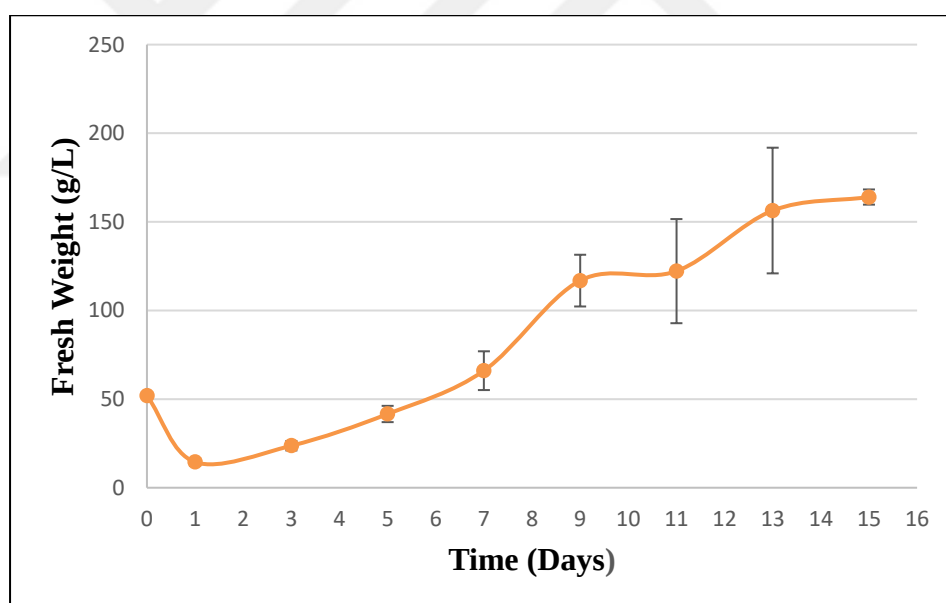


Figure 5.11. Fresh weight changes of *N. tabacum* cv. Xanthi cell suspension cultures in 15 days growth period

When DW changes were examined for *N. tabacum* cv. Xanthi cell suspension cultures, measurements were taken after day 1 so the starting point was 1.37 ± 0.08 g/L (9.46 percent of FW). DW measurements had almost the same type of increase in FW. Also these measurements correlated with pH which can be seen in Figure 5.12, Figure 5.13, and Figure 5.14. On day 15, final DW was 12.98 ± 0.42 g/L (7.91 percent of FW). It can be seen from

these FW and DW measurements that optimal time to subculture the *N. tabacum* suspensions were between day 7 and 9 because FW/DW ratio and suspension viscosity changes after day 9.

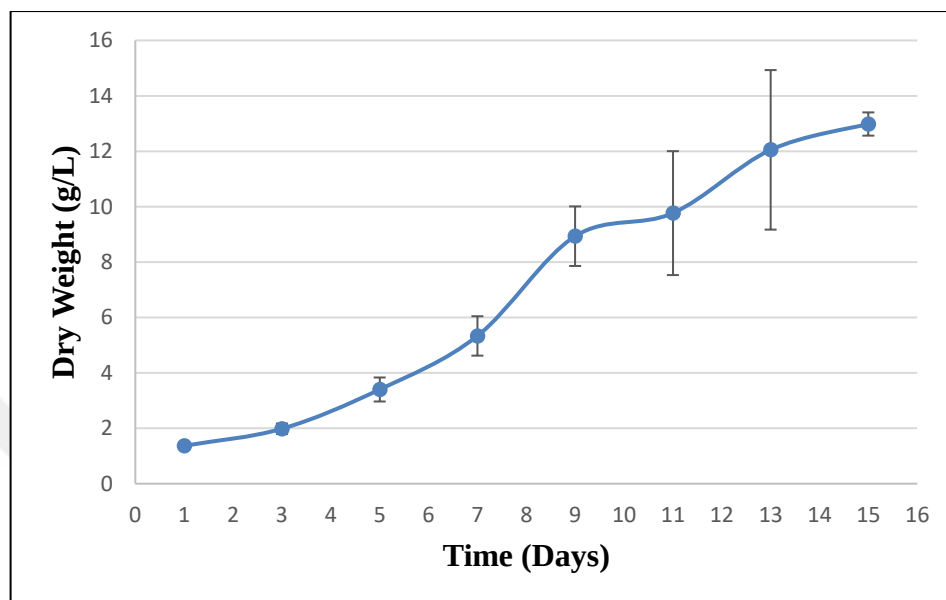


Figure 5.12. Dry weight changes of *N. tabacum* cv. Xanthi cell suspension cultures in 15 days growth period

Sugar consumption was analyzed in this study for 15 days period starting from day 0 using HPLC which can be seen in Figure 5.15. Sucrose, glucose and fructose levels were measured. Since plant cells prefer sucrose as its primary carbon source, they start to hydrolyze sucrose to glucose and fructose immediately by invertase enzyme secretion [83,129]. During this process *N. tabacum* cv. Xanthi cells firstly started to utilize glucose and after that fructose was utilized. Sugar concentrations in day 0 started with 23.56 ± 0.04 g/L sucrose, 3.59 ± 0.03 g/L glucose and 2.07 ± 0.01 g/L fructose. Sucrose was completely hydrolyzed until day 4; however, all of the carbon sources were not finished by the cells. There was still 1.83 ± 0.84 g/L glucose and 4.59 ± 0.92 g/L fructose left in the suspension that cells can utilize in day 15.

According to the changes in growth parameters, *N. tabacum* cv. Xanthi cell suspensions were in log phase between day 1 to 11 and in stationary phase between day 11 to 15. The optimal time to subculture the cells can be determined as the days between 7 and 9 according to the growth parameters and the physical state of the suspension cultures.

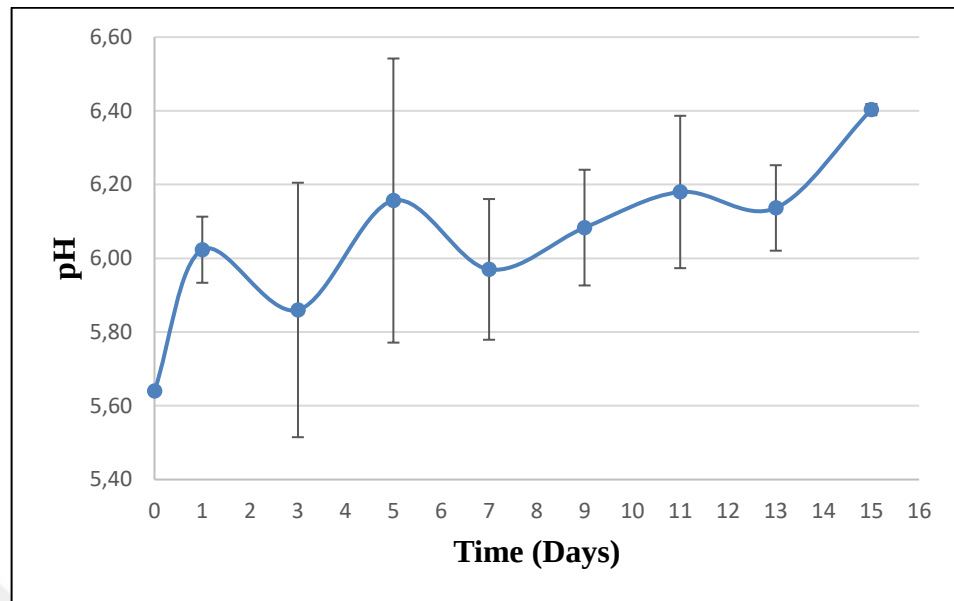


Figure 5.13. pH changes of *N. tabacum* cv. Xanthi cell suspension cultures in 15 days growth period

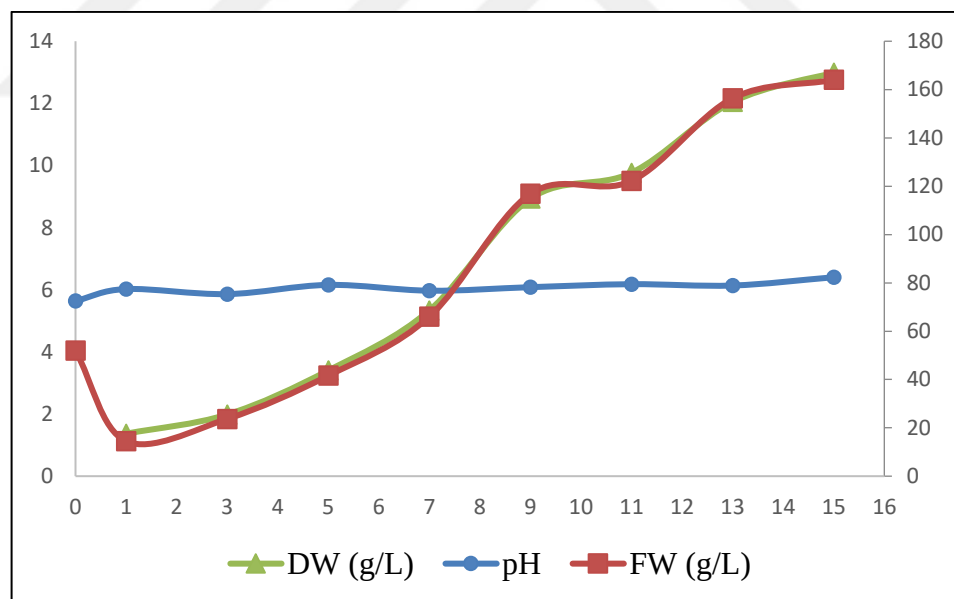


Figure 5.14. Correlation between FW, DW and pH in *N. tabacum* cv. Xanthi cell suspension cultures in 15 days growth period

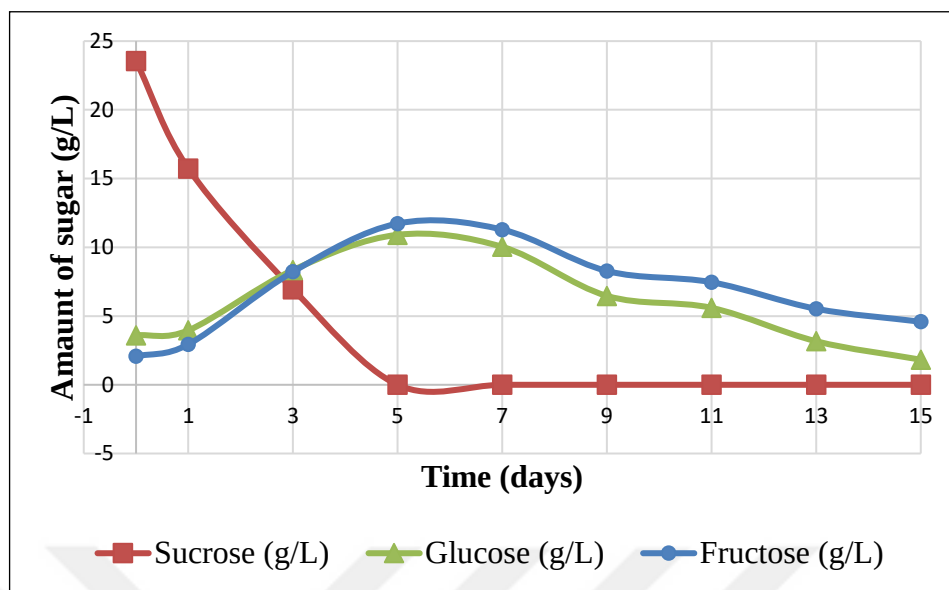


Figure 5.15. Sugar consumption of *N. tabacum* cv. Xanthi cell suspension cultures throughout 15 days growth period

5.4. EXTRACTION AND ANALYSIS OF SOLANESOL FROM *NICOTIANA TABACUM* CV. XANTI CELL SUSPENSION CULTURES

Solanesol standards were prepared by dissolving 10 mg HPLC grade solanesol in 10 ml acetonitrile. Solanesol standards were prepared as 1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml. Different mobile phases were tried according to the literature; however, the best one for this study was Acetonitrile: Isopropanol (80:20) [118]. According to HPLC analysis, retention time (RT) of solanesol was approximately in 6.6 minutes. Retention time correlated with the literature when compared with the column type, injection volume and mobile phase [120,131,132]. HPLC chromatogram, RT table and calibration curve can be seen in Figure 5.16., Table 5.2. and Figure 5.17. Solanesol concentration vs. peak area was used to construct calibration curve and it had R^2 value of 0.9903. Calibration curve equation was $y=6E-0.6x+0.1038$.

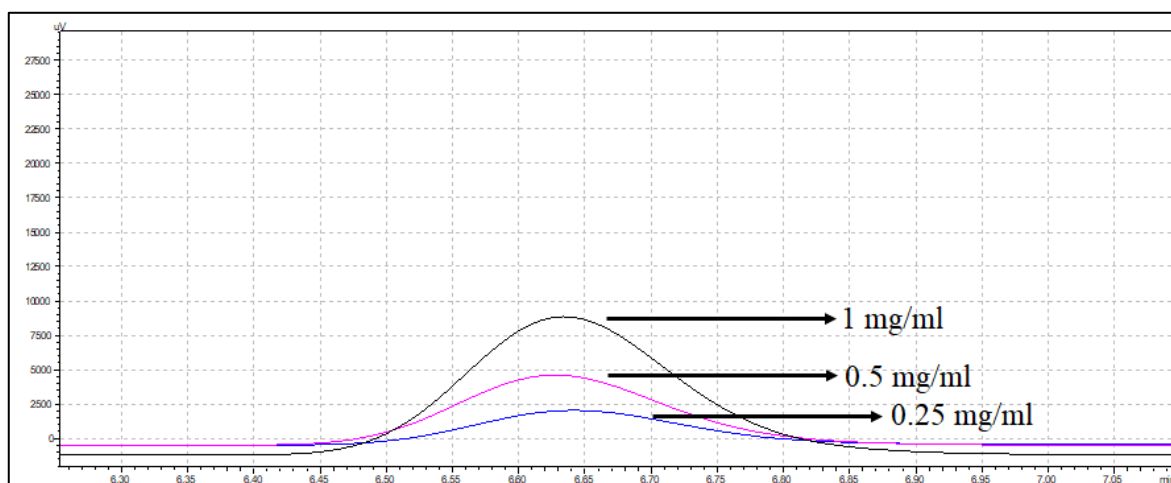


Figure 5.16. HPLC chromatogram of solanesol standards

Table 5.2. Retention time and peak area for solanesol standards

Solanesol standards (mg/ml)	Retention time (RT)	AREA
1	6.630	151348
0.5	6.623	59212
0.25	6.638	29672

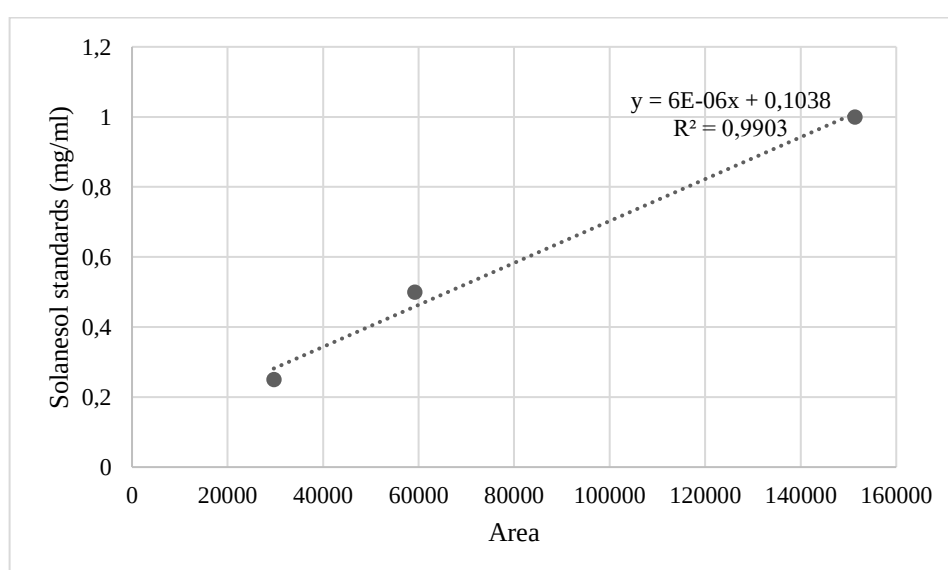


Figure 5.17. Solanesol concentration vs. peak area, calibration curve and equation

In this study, a solanesol extraction protocol for tobacco leaves were used to extract solanesol from *N. tabacum* cv. Xanthi cell suspension cultures [124]. Cells that were collected in multiple subcultures were washed and dried at 65°C for 12 hours. After drying and grinding process saponification reaction was started with EtOH and KOH. Solanesol is generally found as ester-bound in tobacco. Incubating grinded cells with EtOH and KOH in a 60°C water-bath for 4 hours was to ensure hydrolysis of ester-bound solanesol. After this process, a liquid-liquid and a solid-liquid extraction were conducted on the samples. In liquid-liquid extraction, filtrate was mixed with water and then extracted with n-hexane for four times. Since solanesol very low solubility in water, it has a tendency to dissolve in hexane part. This way solanesol can be extracted by solvent partitioning [116,124,132]. In solid-liquid extraction, cell residues were extracted with n-hexane for four times using ultrasonication. Ultrasonication enhances the extraction of solanesol from samples. Liquid-liquid extraction and solid-liquid extraction protocols were separately applied to cells and the HPLC data was obtained before combining the two extractions which showed the positive effect of ultrasonication (Appendix B).

According to the HPLC results of the solanesol extracted from the cells, 9.2883 ± 0.07 mg/ml (0.4644 percent of DW) solanesol was extracted from replicate 1 (R1), 19.8091 ± 0.1 mg/ml (1.9809 percent of DW) from replicate 2 (R2) and 14.5864 ± 0.12 mg/ml (1.4586 percent of DW) from replicate 3 (R3). The average of these three technical replicates was 14.5613 ± 4.71 mg/ml which is the 1.4561 percent of DW. The difference between the replicates can come from the solvent partitioning part of the study since it was difficult to separate phases. HPLC chromatograms, RT and peak area of each injection can be seen in Figure 5.18., Figure 5.19. and Table 5.3.

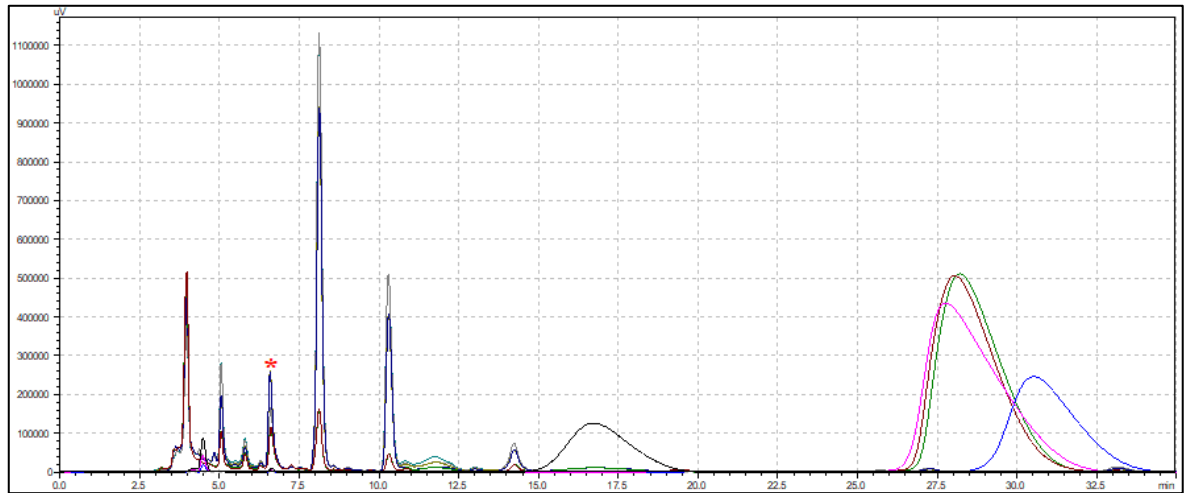


Figure 5.18. HPLC chromatogram of solanesol extraction from *N. tabacum* cv. Xanthi cells (*:solanesol peaks)

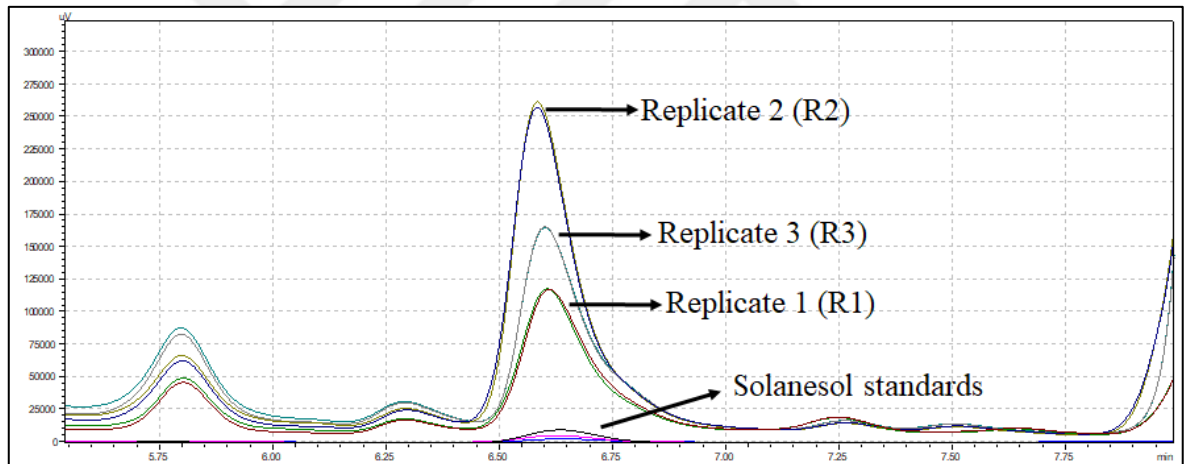


Figure 5.19. Detailed HPLC chromatogram of solanesol extracted from *N. tabacum* cv. Xanthi cells showing each replicate with standards

Table 5.3. Retention time, peak area and concentration (mg/ml) of solanesol extraction from *N. tabacum* cv. Xanthi cells

	Injection	RT	Area	Concentration (mg/ml)
Replicate 1	1	6,607	1541551	9.334624
	2	6,602	1526080	9.241984
Replicate 2	1	6,580	3303063	19.88254
	2	6,581	3278543	19.73572
Replicate 3	1	6,597	2404521	14.50208
	2	6,598	2432700	14.67082

The solanesol concentrations in dry tobacco leaves could not be determined due to the impurities each replicate left on the HPLC column. It can be seen in Figure 5.20. that there were peaks around 6.6 minutes; however, even the two injections of each replicate were far apart from each other. When HPLC chromatogram of the dry tobacco leaves were compared with the solanesol extraction from *N. tabacum* cells, it can be seen that separation between extracted compounds was more clear. Tobacco leaves grown in the field are exposed to different stress factors which induce them to produce high levels of SMs in order to protect themselves from these stress factors. It can be seen in the Figure 5.20. that different compounds with high concentrations were extracted with this protocol from the dry leaves that resulted in unseparated peaks. These compounds can be polyphenols, flavonoids, chlorogenic acid, caffeic acid and rutin which are known to be extracted protocols similar to this. Mixture of these compounds probably resulted in a change of retention time for solanesol so it could not be detected [132]. Tobacco leaf samples require further purification steps to analyze solanesol content.

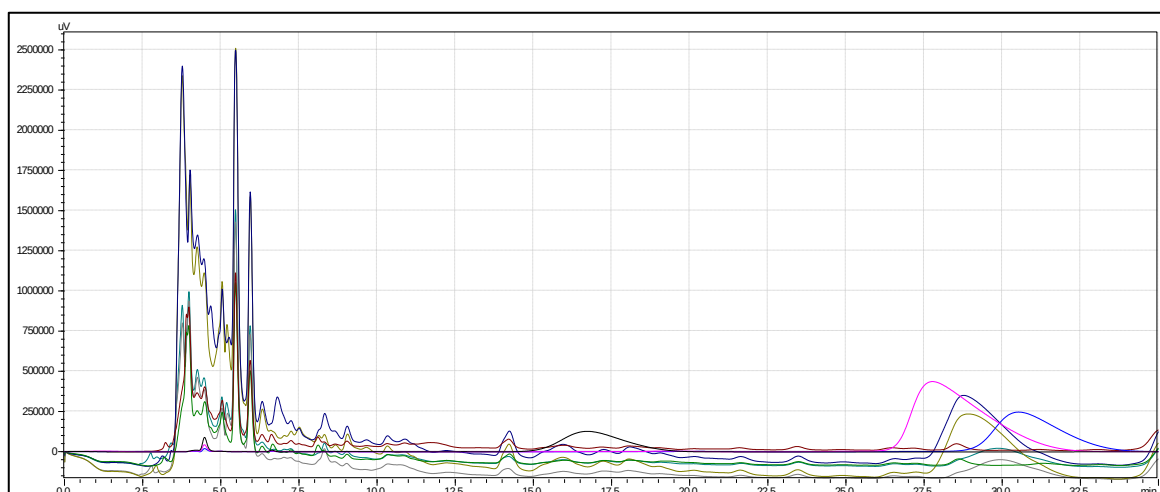


Figure 5.20. HPLC chromatogram of solanesol extraction from dry tobacco leaves

In the literature *N. tabacum* leaves found to contain solanesol between 0.04-3.6 percent of DW. Solanesol concentrations extracted from *N. tabacum* cv. Xanthi cell suspensions were correlating with the literature with the average of 14.5613 mg/ml (1.4561 percent of DW) and standard deviation of 4.71 [8,9,107,116,124,132].

5.5. PROCESS SCALE-UP OF *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURE VIA STIRRED-TANK BIOREACTOR

In this study, a 6 L stirred-tank bioreactor with 3L working volume was used. Media (NtCIM5) and growth temperature (25°C) were not changed throughout these five trials. Parameter changes can be seen in Table 5.4. for the five trials. Parameters of the first trial were determined and modified from the literature [126]. Inoculant size was 80 g (*N. tabacum* cv. Xanthi cells from the suspension culture), air flow was set to 1.29 L/min (3x0.43 vvm), dissolved oxygen was set to be higher than 30 percent and rpm was set to 150. pH was not controlled initially; however, after the first day of batch pH dropped from 5.78 to 5.32 so it was decided to keep at 5.9. Cells started to form clusters around the metal parts of the bioreactor (wall growth) immediately after inoculation. During the first day of the batch, foaming started due to bubble aeration, sucrose and protein rich media and high rpm [133]. In the second day of the batch cells lost their colors and the media started to change color from clear to yellowish brown which is generally a result of phenolic compound production and death of cells due to stress factors [33,66,134]. Batch was ended at day 2 and final mass

of the *N. tabacum* cells decreased from 80 g to 56.04 g. Changes throughout the batch can be seen in Figure 5.21. Sugar consumption in day 1 and 2 can be seen in Figure 5.22.

Table 5.4. Effect of parameter changes in *N. tabacum* cv. Xanthi final cell mass in scale-up studies

# of trials	Temperature (°C)	Stirrer (rpm)	pH control	pO ₂ (%)	Air flow (L/min)	Inoculant size (g)	Batch time (days)	Final cell mass (g)
1	25	150	5.9 (1 day starting the batch)	>30	1.29	80	2	56.04
2	25	150	-	>30	1.29	55	7	52.31
3	25	75	-	>30	1.29	60	3	42.69
4	25	75	6.0	>30	0.6	60	7	245.51
5	25	75	6.0	>30	0.6	60	6	170.57

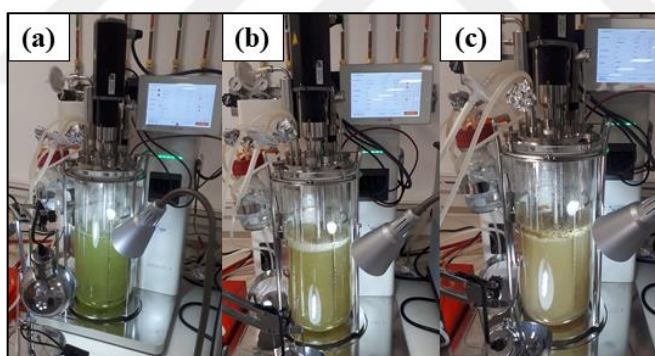


Figure 5.21. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 1 at (a) day 0, (b) day 1, and (c) day 2

In second trial, inoculant size was 55 g (*N. tabacum* cv. Xanthi cells from the suspension culture), air flow was set to 1.29 L/min (3x0.43 vvm), dissolved oxygen was set to be higher than 30 percent and rpm was set to 150. pH was not controlled since plant cells have an ability to control the pH of their environment when all the conditions are optimized [135]. Foaming problem increased and cells were mostly dead in day 3 as can be seen in Figure 5.23. In order to observe furthermore, batch was continued until day 7. As can be seen in Figure 5.24., normally *N. tabacum* cells in flasks that are growing normally use up the carbon

sources (Figure 5.15.) in order to grow; however, most of the sugars were still present in the media at high amounts at day 7 which also shows that cells were mostly dead. Also final cell mass was decreased to 52.31 g which shows the decomposition of the cells. *N. tabacum* cells in the bioreactor were affected by the shear stress caused by the Rushton turbine impeller and high oxygenation.

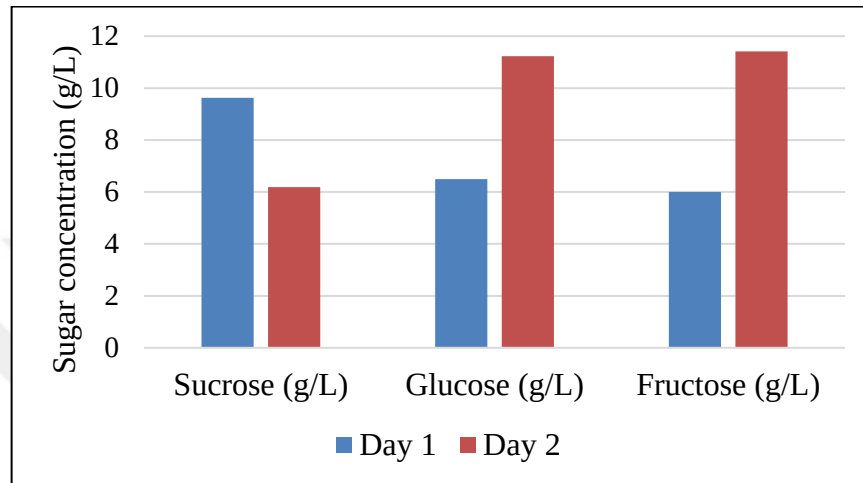


Figure 5.22. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 1 sugar concentrations in day 1 and 2

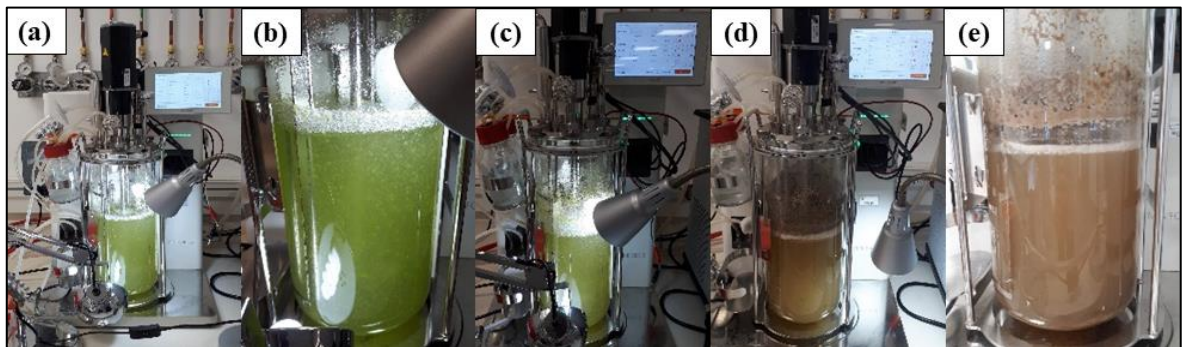


Figure 5.23. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 2 at (a) day 0, (b) day 1, (c) day 2, (d) day 3, and (e) day 7

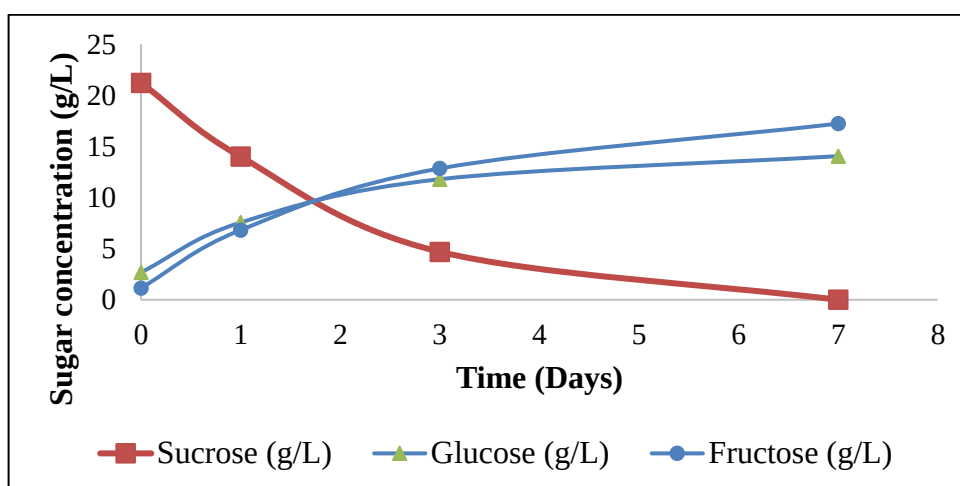


Figure 5.24. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 2 sugar concentration changes in 7 days

In third trial, inoculant size was 60 g (*N. tabacum* cv. Xanthi cells from the suspension culture), air flow was set to 1.29 L/min (3x0.43 vvm), dissolved oxygen was set to be higher than 30 percent and rpm was set to 75. pH was not controlled since plant cells have an ability to control the pH of their environment when all the conditions are optimized [135]. Decreasing the rpm lowered the shear stress; however, air bubbles pushed the cells further up and foaming increased. *N. tabacum* cells were pushed further by the foam which resulted in them sticking into the walls of the reactor without any media around them. The changes in trial three can be seen in Figure 5.25. Cells started to lose their color and media started to change color in day 2. Batch was ended in day 3 and final mass of the cells decreased to 42.69 g. Carbon sources were still present in the medium and even sucrose was not hydrolyzed mostly in day 3 (Figure 5.26.). So the death of the cells were not dependent on the media composition and lack of carbon source, it was dependent on the stress factors caused by the changing pH, high oxygenation and shear stress.

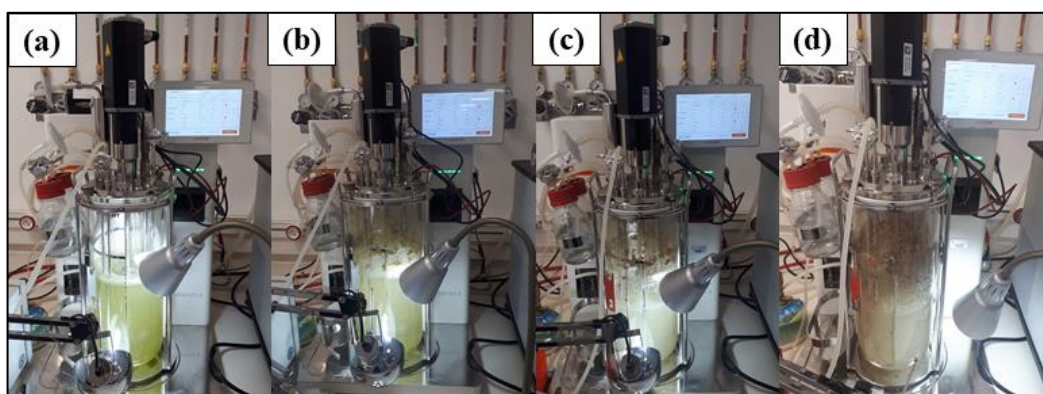


Figure 5.25. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 3 at (a) day 0, (b) day 1, (c) day 2, and (d) day 3

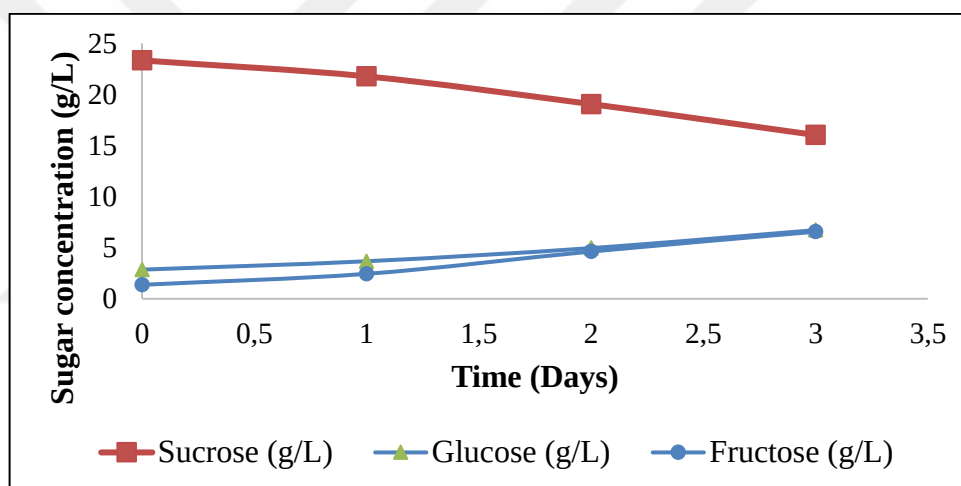


Figure 5.26. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 3 sugar concentration changes in 3 days

In trial four and five, inoculant size was 60 g (*N. tabacum* cv. Xanthi cells from the suspension culture), air flow was set to 0.6 L/min (3x0.2 vvm), dissolved oxygen was set to be higher than 30 percent and rpm was set to 75. pH was controlled at 6 which was determined according to the growth curve analysis of *N. tabacum* cell suspension cultures. Decreasing the air flow and rpm with the stabilized pH resulted in a successful growth (Figure 5.27. , Figure 5.28. and Figure 5.29.). The only difference between fourth and fifth trial was the batch time. In the fourth trial, batch time was 7 days and final cell mass was 245.51 g which was the 4.09 times the initially inoculated amount. In the fifth trial, batch time was 6 days and final cell mass was 170.57 g which was the 2.84 times the initial

inoculant. When sugar consumption in bioreactor compared to the flasks, they were similar. In this study, *N. tabacum* cells were grown in a 6 L stirred-tank bioreactor (3 L working volume) with similar growth kinetics to cell suspension cultures in flasks.

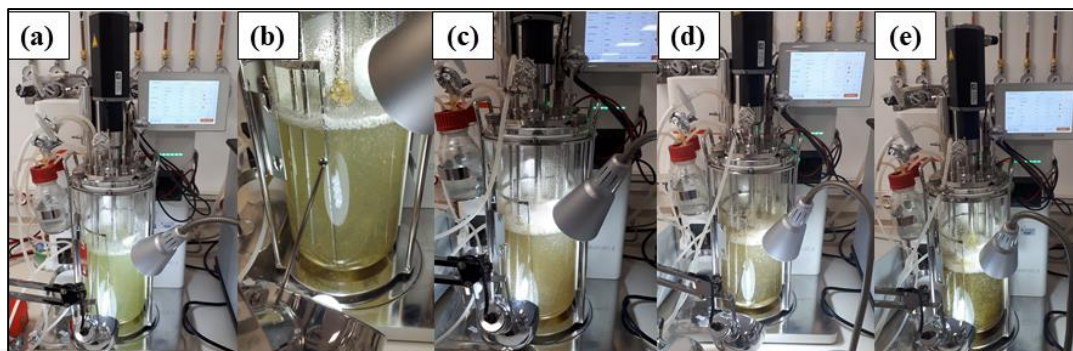


Figure 5.27. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 4 at (a) day 0, (b) day 1, (c) day 3, (d) day 5, and (e) day 7

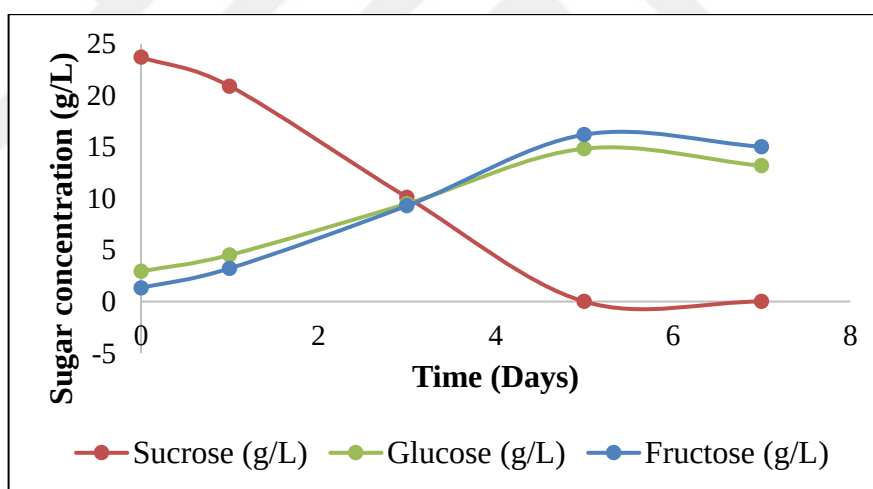


Figure 5.28. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 4 sugar concentration changes in 7 days

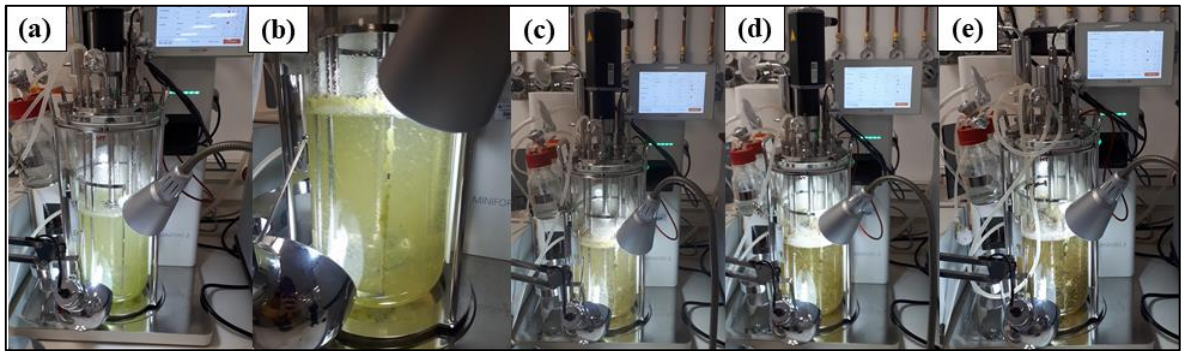


Figure 5.29. *Nicotiana tabacum* cv. Xanthi cell suspension culture bioreactor trial 5 at (a) day 0, (b) day 1, (c) day 3, (d) day 5, and (e) day 6

6. CONCLUSION AND FUTURE PROSPECTS

Nicotiana tabacum cv. Xanthi seeds had 90.32 percent germination rate in the presence of MS, sucrose, casein hydrolysate and charcoal. Callus induction rate from germinated leaves was determined as 100 percent in the presence of NAA and BAP. Cell lines, newly initiated callus and micropropagations were maintained throughout the study.

N. tabacum cv. Xanthi cell suspension cultures were initiated and maintained for 21 subcultures successfully in the presence of MS, sucrose, casein, NAA and BAP. Growth rate of the suspensions were in the average of 20.588 ± 5.9 gFW/L.D between 8th and 21th subcultures. Cell viability during maintenance was determined through FDA test by the green fluorescence of the living cell and structures of the cells were observed in light microscope. Since the *N. tabacum* cv. Xanthi cell suspension culture maintenance protocol was optimized in this study, these cultures can be used in other secondary metabolite production studies and can be a foundation for transgenic studies.

Growth parameters of the *N. tabacum* cv. Xanthi cell suspension cultures were determined by the fresh weight, dry weight, pH and sugar consumption analysis. Cell were in log phase between days 1 to 11 and in stationary phase between days 11 to 15. Optimal pH for the growth of the cells were determined as 6 and the subculture period was determined as 7 to 9 days due to suspension culture media viscosity and growth parameters.

Solanesol extraction from the *N. tabacum* cv. Xanthi cells coming from the suspension cultures and dry tobacco leaves were established through combination of solid-solid and solid-liquid extraction which was enhanced with the ultrasonication. Solanesol extracted from the cells were between 0.4644 and 1.9809 percent of the dry weight with the average of 1.4561 percent of DW. Solanesol content of the leaves were not possible to determine due to impurities and the samples required further purification steps. This study showed that solanesol can be extracted from the *N. tabacum* cv. Xanthi cell suspension cultures for the first time in literature.

Scale-up parameters of *N. tabacum* cv. Xanthi cell suspension culture was determined with different parameter changes in a bioreactor. 25°C, 75 rpm, pH set at 6.0, dissolved oxygen higher than 30 percent, 0.6 L/min air flow, 60 g inoculant size and batch time of 7 days were

determined as optimal scale-up parameters for *N. tabacum* cv. Xanthi cell suspension cultures with 4.09 times increase in the final inoculant size.

Since maintenance of *N. tabacum* cv. Xanthi cell suspension cultures, solanesol extraction and scale-up parameters were optimized, this study can be a strong reference to large-scale production of solanesol and other secondary metabolites from cell suspension cultures through bioreactors.

In future studies, tobacco mosaic virus or hydrogen peroxide can be used as elicitors to enhance the solanesol content in *N. tabacum* cv. Xanthi cell suspension cultures. Bioreactor trials were preliminary studies so the parameters can be optimized further for higher biomass accumulation. This way solanesol that is used in pharmaceutical industry can be produced in high levels in less time and space via bioreactors.

As a consequence, this study will be a foundation for the large-scale production of high purity solanesol from tobacco cell suspension cultures which is the main future goal.

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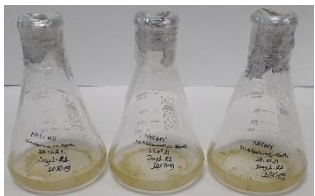
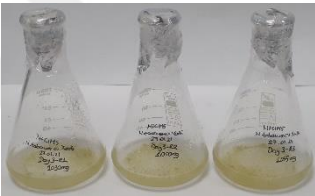
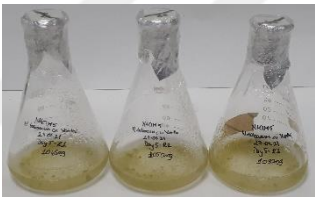
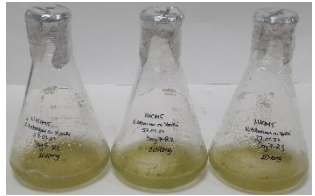
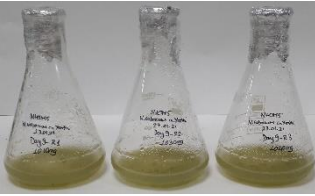
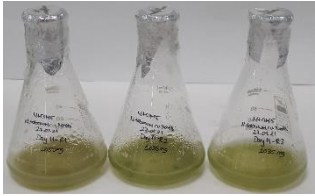
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APPENDIX A: GROWTH CURVE ANALYSIS RESULTS IN DETAIL

	Replicate 1 (R1)	Replicate 2 (R2)	Replicate 3 (R3)		R1	R2	R3	Average	Standard deviation
Day 1				FW (g/L)	12.65	14.05	16.75	14.48	2.08
				DW (g/L)	1.30	1.35	1.45	1.37	0.08
				pH	6.08	5.92	6.07	6.02	0.09
Day 3				FW (g/L)	22.50	21.85	26.75	23.70	2.66
				DW (g/L)	1.90	1.85	2.20	1.98	0.19
				pH	6.21	5.52	5.85	5.86	0.35
Day 5				FW (g/L)	39.05	38.85	46.90	41.60	4.59
				DW (g/L)	3.15	3.15	3.90	3.40	0.43
				pH	6.55	5.78	6.14	6.16	0.39
Day 7				FW (g/L)	78.65	60.25	59.20	66.03	10.94
				DW (g/L)	6.15	5.00	4.85	5.33	0.71
				pH	6.15	5.77	5.99	5.97	0.19
Day 9				FW (g/L)	100.25	127.65	122.65	116.85	14.59
				DW (g/L)	7.75	9.85	9.20	8.93	1.08
				pH	6.03	5.96	6.26	6.08	0.16
Day 11				FW (g/L)	134.00	143.85	88.75	122.20	29.38
				DW (g/L)	10.80	11.30	7.20	9.77	2.24
				pH	6.40	6.15	5.99	6.18	0.21

Day 13		FW (g/L)	134.70	137.15	197.30	156.38	35.46
		DW (g/L)	10.05	10.75	15.35	12.05	2.88
		pH	6.06	6.08	6.27	6.14	0.12
Day 15		FW (g/L)	167.05	159.10	165.85	164.00	4.29
		DW (g/L)	12.50	13.20	13.25	12.98	0.42
		pH	6.40	6.42	6.39	6.40	0.02

APPENDIX B: RESULTS OF PREVIOUS SOLANESOL EXTRACTION

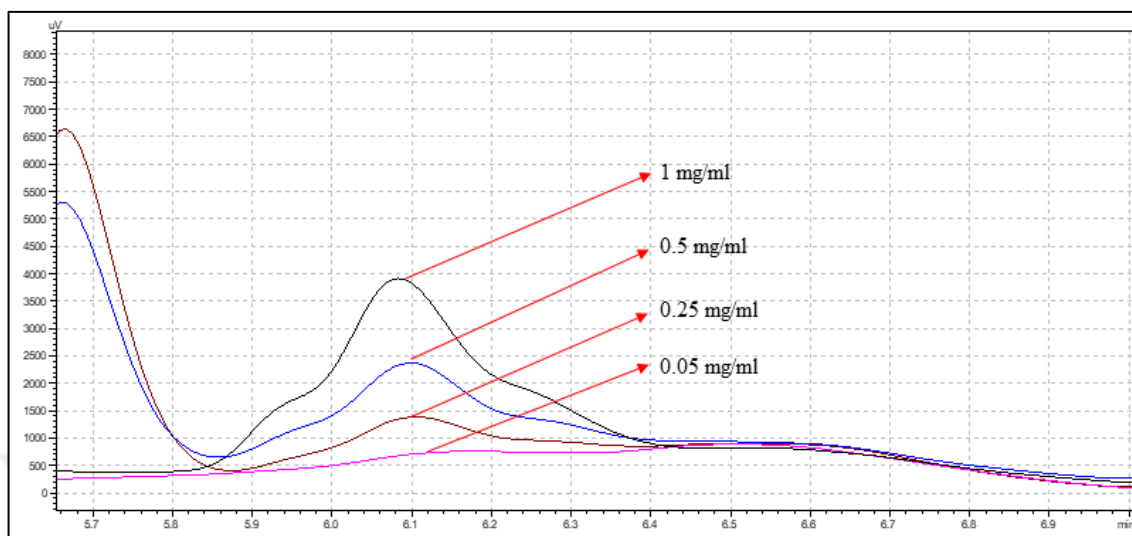


Figure B.1. HPLC chromatogram of solanesol standards.

Table B.1. Retention time and peak area for solanesol standards.

Solanesol standards (mg/ml)	Retention time (RT)	AREA
1	6.079	91517
0.5	6.095	63425
0.25	6.101	34565
0.05	6.170	26650

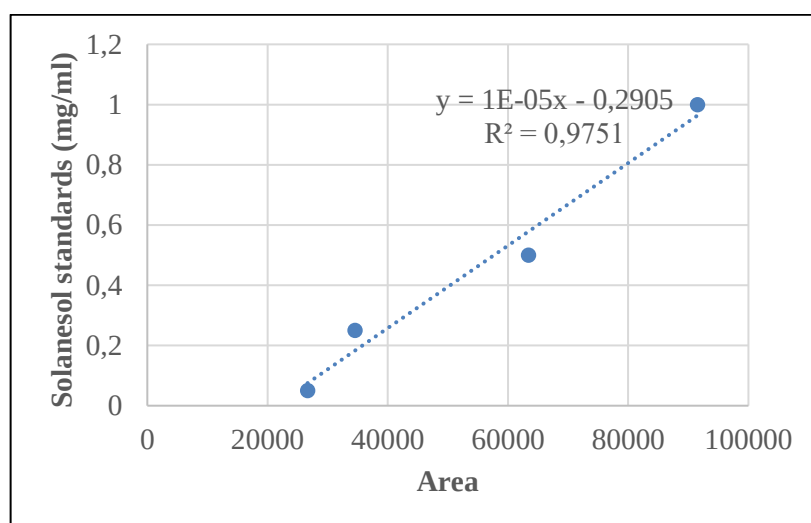


Figure B.2 .Solanesol concentration vs. peak area, calibration curve and equation.

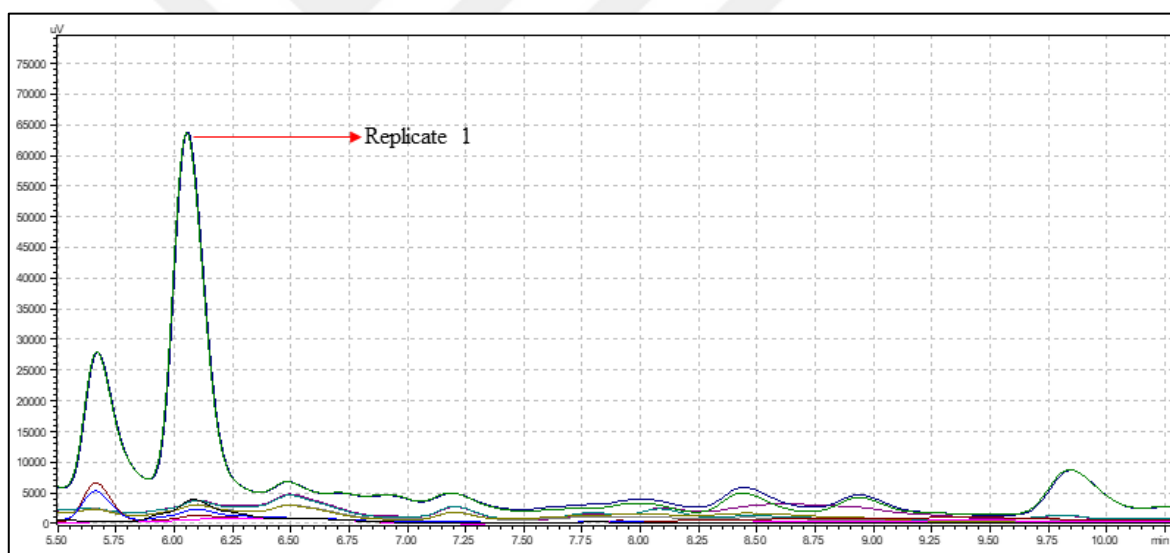


Figure B.3. HPLC chromatogram of liquid-liquid extraction of solanesol from *N. tabacum* cv. Xanthi cells.

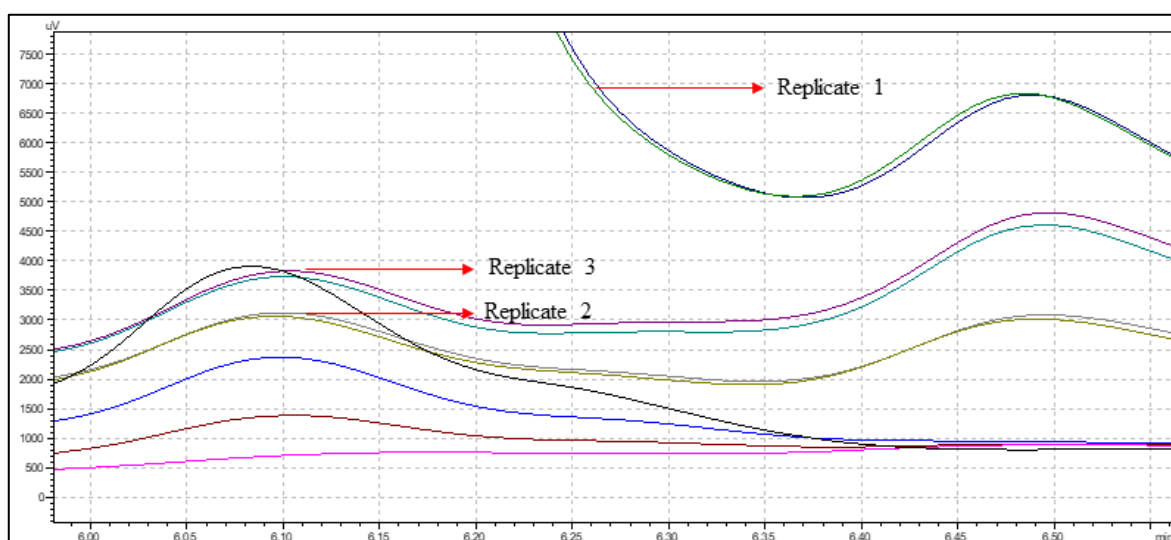


Figure B.4. Detailed HPLC chromatogram of liquid-liquid extraction of solanesol from *N. tabacum* cv. Xanthi cells.

Table B.2. Retention time, peak area and concentration (mg/ml) of solanesol liquid-liquid extraction from *N. tabacum* cv. Xanthi cells.

	Injection	RT	Area	Concentration (mg/ml)
Replicate 1	1	6.048	707836	9.409
	2	6.053	706815	9.396
Replicate 2	1	6.091	68246	0.645
	2	6.095	68565	0.649
Replicate 3	1	6.096	70651	0.678
	2	6.099	73912	0.722

Table B.3. Retention time, peak area and concentration (mg/ml) of solanesol solid-liquid extraction from *N. tabacum* cv. Xanthi cells.

	Injection	RT	Area	Concentration (mg/ml)
Replicate 1	1	6.047	626280	8.292
	2	6.048	624351	8.265
Replicate 2	1	6.052	771678	10.284
	2	6.052	772234	10.292
Replicate 3	1	6.055	708631	9.420
	2	6.055	706403	9.390