PRODUCTION OF RESVERATROL FROM *VITIS* (L.) PLANT CELL SUSPENSION CULTURE USING STIRRED TANK REACTOR

by

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ABSTRACT

PRODUCTION OF RESVERATROL FROM *VITIS* (L.) PLANT CELL SUSPENSION CULTURE USING STIRRED TANK REACTOR

Plant cell suspension culture (PCSC) are favorable for scaling up production due to their faster growth cycles, the opportunity to control the supply of nutrients and the high degree of uniformity between individual cells. Hence, the purpose of the study was to (1) conceptually design a process applicable to t-resveratrol production using *V. labrusca* (L.) cv. Isabel and *V. vinifera* (L.) cv. Öküzgözü species, and (2) optimize the parameters such as the media composition, the aeration rate, the speed of mixing, and the use of elicitors for the scale-up process. To the best of our knowledge, this is the first study performed on the optimization of plant cell suspension culture, scale-up studies, and t-resveratrol production using *Vitis labrusca* (L.) cv. Isabel. Methyl jasmonate (MeJA-100µM) and ß-Cyclodextrin (ß-CD-50mM) were introduced into CSCs as elicitors. The biomass of both species, when cells were treated with MeJA, decreased based on the biomass reached in control cells. The results indicated that they could not work synergistically to improve the production of t-resveratrol. The highest amount of t-resveratrol in *V. labrusca* cv. Isabel CSC was found to be 6.78 mg/L (intracellular) and 28.23 mg/L (extracellular), whereas in *V. Vinifera* cv. Öküzgözü CSC it was found to be 84.8 mg/L (extracellular) via HPLC. *V. labrusca* cv. Isabel cells were scaled up from shake-flasks to a 6 L stirred tank reactor (STR) under the controlled conditions of batch culture. Setting the mixing to 75 rpm, and the initial aeration rate to 0.02 vvm and with the presence of casein hydrolysate (CH) in media led to the highest biomass of 11.8 g DW/3L (5^{th} day) representing 4.52-fold higher biomass from the initial inoculation, whereas without CH in media, the highest biomass was revealed as 13.1 g DW/3L (8^{th} day) representing 5.02-fold increase. Experiments conducted in a flask with CH in the media revealed the biomass increase to 14.57 g DW/L (11^{th} day), whereas without CH, 14.48 g DW/L (9^{th} day) biomass was obtained. t-resveratrol production in STR with or without CH supplementation was found to be around 4 mg/L and 6 mg/L respectively. Besides, in response to light, accumulation of biomass and t-resveratrol were found to be 300 g FW /3L and 23.26 mg/L respectively. This study based on improvement in resveratrol production from *Vitis* CSC will shed light on future studies.

ÖZET

VİTİS (L.) BİTKİ HÜCRE SÜSPANSİYON KÜLTÜRÜNDEN KARIŞTIRMALI TANK REAKTÖRÜ İLE RESVERATROL ÜRETİMİ

Bitki hücre süspansiyon kültürleri, daha hızlı büyüme döngüleri, besin elementlerini kontrolü ve hücrelerin yüksek derecedeki benzerliği nedeniyle üretimde ölçek büyütme için uygundur. Dolayısıyla bu çalışmada; (1) *V. labrusca* (L.) cv. Isabel ve *V. vinifera* (L.) cv. Öküzgözü türleri kullanılarak, t-resveratrol üretiminde uygulanabilir bir prosesin kavramsal olarak tasarlanması ve (2) ölçek büyütme işlemi için besiyeri, havalandırma hızı, karıştırma hızı ve elisitörlerin kullanımı gibi parametrelerin optimize edilmesi amaçlandı. Bilgimiz dahilinde, bu çalışma, *V. labrusca* (L.) cv. Isabel kullanılarak, bitki hücre süspansiyon kültürünün optimizasyonu, ölçek büyütme çalışmaları ve t-resveratrol üretimi üzerine yapılan ilk çalışmadır. Metil jasmonat (MeJA-100µM) ve ß-Siklodekstrin (ß-CD-50mM), hücre süspansiyon kültüründe elisitör olarak kullanıldı. Her iki türde biyokütlenin, hücreler MeJA ile indüklendiğinde, kontrol hücrelerinde ulaşılan biyokütleye göre daha az olduğu gösterildi. MeJA ve ß-CD elisitörlerinin, t-resveratrol üretimini arttırmak için sinerjik olarak çalışmadığı sonucuna varıldı. En yüksek t-resveratrol miktarının HPLC ile *V. labrusca* cv. Isabel hücre süspansiyon kültüründe 6.78 mg/L (hücre içi) ve 28.23 mg/L (hücre dışı) olduğu bulunurken, *V. Vinifera* cv. Öküzgözü kültüründe ise 84,8 mg/L (hücre dışı) olarak bulundu. *V. labrusca* cv. Isabel hücreleri, kontrollü kültür koşulları altında erlenden 6 L'lik karıştırmalı tanklı biyoreaktöre ölçeklendirildi. Karıştırmanın 75 rpm'ye ve havalandırma oranının 0,02 vvm'ye ayarlanması ve ortamda kazein hidrolizat (KH) bulunması ile ilk inokülasyondan 4,52 kat daha yüksek olan 11,8 g DW/3L'lik (5. gün) en yüksek biyokütle elde edildi. Aynı şartlar altında, KH içermeyen kültürde ise 5,02 kat artışla 13,1 g DW/3L (8. gün) olarak en yüksek biyokütle üretimi sağlandı. Erlen içerisinde yapılan çalışmalarda, besiyerinde KH bulunması, biyokütleyi 14.57 g DW/L'ye (11. gün) çıkarırken, KH olmadan biyokütle miktarı 14.48 g DW/L (9. gün) olarak elde edildi. Karıştırmalı tanklı biyoreaktörde t-resveratrol üretimi, besiyerinde KH ilavesi olan veya olmayan, sırasıyla 4 mg/L ve 6 mg/L civarında bulundu. Işığa yanıt olarak ise hücre kültüründen, 300 g FW/3L biyokütle ve 23,26 mg/L t-resveratrol üretimi sağlandı. *Vitis* hücre süspansiyon kültüründen t-resveratrol üretimini artırmaya dayalı bu çalışma, ilerideki çalışmalara ışık tutacaktır.

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μ	Specific growth rate
Ag	Silver
$AlCl_3$	Aluminum chloride
$AgNO_3$	Silver nitrate
BAP	6-benzyl amino purine
B5	Gamborg's B-5 Basal Medium with vitamins
Cd	Cadmium
CDs	Cyclodextrins
CH	Casein hydrolysate
$CaCl_2$	Calcium chloride
$CdSO_4$	Cadmium sulfate
Cu	Copper
$CuCl_2$	Copper chloride
CSC	Cell suspension culture
CSTR	Continuous stirred tank reactor
$CoCl_2$	Cobalt chloride
$CdCl_2$	Cadmium chloride
CHI	Chitosan
dH_2O	Distilled water
DO	Dissolved oxygen
DIMEB	Dimethyl-βeta –cyclodextrins
DW	Dry weight
2.4-D	2.4-dichloro phenoxy-acetic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
EtOH	Ethanol
FDA	Fluorescein diacetate
FW	Fresh weight
GA	Gibberellic acid
GLU	β-glucan
HPLC	High-performance liquid chromatography

HCl	Hydrogen chloride
IAA	Indole-3-acetic acid
In-Ile	Indanoyl-isoleucine
IS	Insect sativa
JA	Jasmonic acid
KH_2PO_4	Potassium dihydrogen phosphate
KNO_3	Potassium nitrate
KI	Potassium iodide
Kinetin	N-2- furamylmethyl -1H-purine-6 Amine
k_La	Volumetric mass transfer coefficient
LS	Linsmaier and Skoog Medium
LED	Light emitting diode
Lin-Gln	N-linolenoyl-l-glutamine
$MgSO_4$	Magnesium sulfate
MS	Murashige & Skoog Basic Medium with vitamins
MeOH	Methanol
MeJA	Methyl jasmonate
MCoA	Malonyl coenzyme A
MeSA	3-methyl-salicylic acid
MeCD	Methyl-β-cyclodextrins
NAA	Naphtalene-acetic acid
NaOCl	Sodium hypochlorite
NH_4NO_3	Ammonium nitrate
Ni	Nickel
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PCC	Plant cell culture
PID	Proportional–integral–derivative (PID) control mechanism
PTC	Plant tissue culture
PCs	Plant cells
PCSC	Plant cell suspension culture
PCV	Packed cell volume
PVC	Polyvinyl chloride

PSMs	Plant secondary metabolites
RDR	Rotating drum reactor
ROS	Reactive oxygen species
SA	Salicylic acid
SM	Secondary metabolite
SMs	Secondary metabolites
t-resveratrol	trans-resveratrol
t-R	trans-resveratrol
UV	Ultraviolet

1. INTRODUCTION

Plant secondary metabolites (PSMs) are being used as medicaments for a very long time, and now, they are utilized in pharmacy, cosmetics, chemistry, and as nutraceutical compounds. For a very long period, field culture of medicinal plants has been used to produce SMs. However, some plants cannot withstand large field culture due to pathogen sensitivity, biotic and abiotic stress, time, and space. This led to taking into account plant cell culture (PCC) as an appropriate technique for SMs production in higher amounts when compared with their plant parents. The cultivation of plant calli and organ culture in liquid media has contributed significantly to current biotechnology considering the production of substances with high commercial value. Callus initiation is the first step in PCC investigations using *in vitro* techniques with the best medium used for cultivation and the suitable genotype and explant. An obtained callus is used to achieve PCSC. PCSC is a suitable technique that can be applied to enhance product yield and it allows for kinetics studies of biomass and SMs production. Through PCSC, various strategies can be applied to enhance SMs production, but elicitation is commonly one of the largest effective. To establish any form of the biotechnological process, the scale-up of plant cells (PCs) in the bioreactor is required. The bioreactor system application for plant cell cultivation is an active field whose application holds great promise for use in the pharmacy and food industry.

The aim of this study is the optimization of conditions using shake flasks and Stirred Tank Reactor (STR) system for biomass and SM production. *V. vinifera* cv. Öküzgözü */V. labrusca* cv. Isabel cell lines and t-resveratrol were used as a reference (model) plant and SM respectively, for the optimization of the system. Tissue culture conditions of selected *Vitis vinifera* cv. Öküzgözü */Vitis labrusca* cv. Isabel's variety has been optimized first. The current protocols in the literature were used for our selected species and it has been modified further. *Vitis vinifera* cv. Öküzgözü */Vitis labrusca* cv. Isabel cell suspension culture (CSC) has been grown in a shake flask with optimized conditions. *Vitis labrusca* cv. Isabel cell suspension has been transferred in Stirred Tank Reactor (STR) system. Culture parameters such as nutritional requirement, inoculum density, mixing, dissolved oxygen pH, and temperature have been studied and optimized in STR for biomass growth and t-resveratrol production.

Firstly, the bioreactor contained nutrient-rich media suitable for the growth of biomass, then elicitors have been added for the induction of t-resveratrol production. Intra and Extracellular t-resveratrol were analyzed in suspension culture since t-resveratrol is either secreted or not secreted into the medium.

Consumption of t-resveratrol reported from many studies (1) (2) (3) prevents cardiovascular diseases, neurodegenerative diseases, and different types of cancers and its role as an antioxidant and an antibacterial. It displays antifungal and anti-microbial activities as well. *Vitis* (L.) species are a suitable plant to induce t-resveratrol biosynthesis after elicitation. Nevertheless, the production of t-resveratrol and its derivatives in great quantity by using chemical synthesis or plant extraction has limitations; therefore, biotechnology may show a powerful route for scaling up the production of this compound. By using PCSC and engineered microorganisms, t-resveratrol can be produced. PCSCs have an advantage over microorganisms in that they can naturally produce t-resveratrol in response to elicitors without needing genetic modification of PCs.

In addition to the many biological functions of t-resveratrol and the present uses it has, it is important to show up its presence in the pharmacy and cosmetic industries. Production of t-resveratrol through bioreactors and using of elicitors should be considered a considerable route of its biosynthesis.

2. LITERATURE SURVEY

Biotechnology possesses a great potential to utilize tissues, cells, organs, or whole organisms by cultivating them *in vitro* and genetically manipulating them to achieve desired compounds (4). Plant-based compounds are being used in developed countries, as well as developing countries. Plants are the most contributors to disease treatment despite the use of synthetic chemistry as a technique to identify and produce medications. Isolation of PSM obtained from their natural pathway is still the only viable method due to the complexity of plant structures and substantial efforts to achieve partial or whole chemical synthesis (5). Eighty percent of the 30,000 or more known natural products that are necessary for food, medicine, and industry are of plant origin. The look for new plant-bioactive compounds should in this manner be important in the present and future endeavors toward practical protection based on the utilization of biodiversity (6). Haberlandt, a German physiologist first mentioned the concept of PTC and attempted the growth of tissues, organs, and PCs in a simple nutrient medium, in 1902 (7). PTC refers to the growth and increase of tissues, cells, and organs on synthetic solid or liquid media under controlled and aseptic conditions. In the beginning, the PTC goal was as a research tool for small, isolated parts of plant tissues or isolated cell cultivation. In the half of the 20th century, the idea that plants could be propagated through callus or organ culture became widely accepted, and afterward, the application in the plant propagation industry started (8)(9). PTC technology has diverse applications including the production of SMs, fast multiplication of different plants, production of doubled haploids, disease-free plants, and clonal propagation (10) (11) (12) (13) (14) (15).

2.1. SECONDARY PLANT METABOLITES

SMs are small molecules that act as defense chemicals to defend plants from any environmental deterioration (16). They also have a role in defense against UV-B exposure and the relation of the plants with other organisms (17). Their absence does not inhibit the fundamental processes in plants since they are not required for primary metabolism (18). Considering their biosynthetic origin, PSMs are divided into three main groups: nitrogen-containing SMs, phenolic compounds, and terpenoids (Figure 2.1) (15) (16) (17).

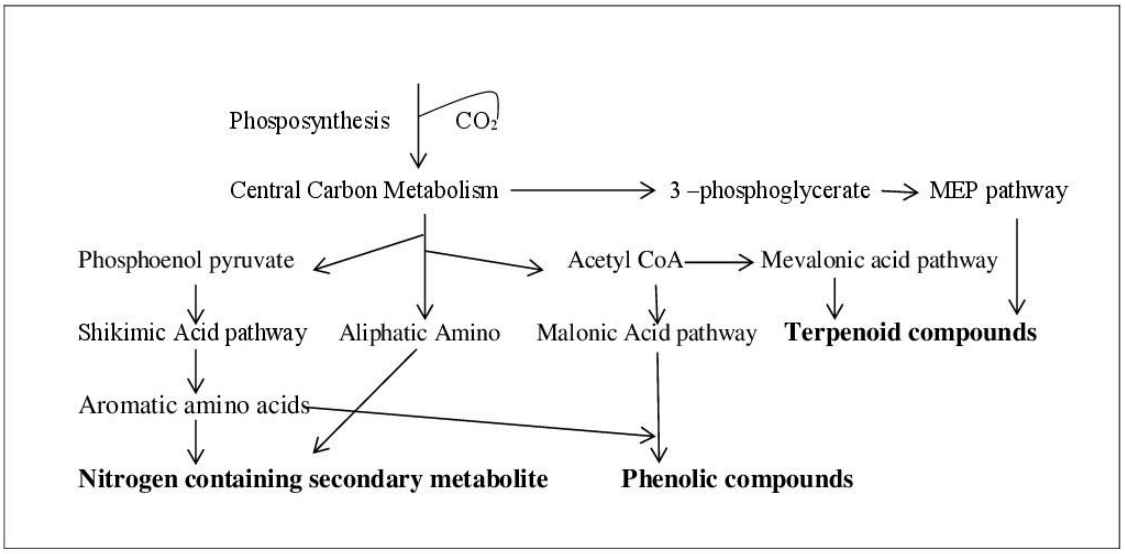


Figure 2.1. The pathway of PSMs biosynthesis (19)

Many articles have reported on SMs production through PCSC (Table 2.1). For instance, *Thalictrum minus* CSC has produced berberine an antibacterial compound. The suspension culture of *H. Niger* L. has produced hyoscyamine which has an anticholinergic role (20) (21).

Some PCC can produce higher amounts of SMs than their plant parents. For example, ginsenoside produced from *Panax ginseng* was 27 % of cell dry weight in culture and 4.5 % in whole plants, shikonin produced from *Lythospermum erythrorhizon* in culture (12%) than in plants (1.5 %) and Anthraquinones produced from *Morinda citrifolia erythrorhizon* in culture (18%) than in plants (2.2 %) (20) (21) (22).

Table 2.1. Production of SMs through PCSC (6) (13).

Plant name	**Secondary metabolite**	**References**
Echium italicum L	Shikonin	(23)
Arnebia sp.	Napthoquinone (shikonin)	(24)
Ailanthus altissima	Canthinone alkaloids	(25)
Aloe saponaria	Tetrahydro anthracene glucosides	(26)
Anchusa officinalis	Rosmarinic acid	(27)
Azadirachta indica	Azadirachtin	(28) (29)

Brucea javanica (L.) Merr.	Canthinone alkaloids	(30)
Camellia Sinensis	Theanine, γ-glutamyl derivatives	(31)
Capsicum annuum L.	Capsaicin	(32)
Cassia acutifolia	Anthraquinones	(33)
Catharanthus roseus	Indole alkaloids	(34) (35)
Catharanthus roseus	Catharanthine	(36) (37) (38)
Cayratia trifoliata	Stilbenes	(39)
Cecropia obtusifolia	Chlorogenic acid Isoorientin hypoglycemic	(40)
Choisya ternata	Furoquinoline alkaloids	(41)
Coleus blumei	Rosmarinic acid	(42)
Chrysanthemum cinerariaefolium	Chrysanthemic acid and pyrethrins	(43)
Cinchona succirubra	Anthraquinones	(44)
Cornus kousa	Polyphenols	(45)
Coffea Arabica	Caffeine	(46)
Coptis japonica	Berberine	(47)
Cruciata glabra	Anthraquinones	(48)
Daucus carota	Anthocyanin	(49)
Digitalis purpurea L.	Cardenolides	(50)
Dioscorea deltoidea	Diosgenin	(51)
*Ephedra s*pp.	L- Ephedrine D-Pseudoephedrine	(52)
Cecropia Obtusifolia	Chlorogenic acid	(40)
Eurycoma longifolia Jack	Cathinone	(53)
Fumaria capreolata	Isoquinoline alkaloids	(54)
Ginkgo biloba	Ginkgolide A	(55)
Glehnia littoralis	Furanocoumarin	(56)
Hyssopus officinalis	Triterpenes; Sterols	(57)
Isoplexis isabellina	Anthraquinones	(58)
Linum flavum L.	5-Methoxypodophyllotoxi	(59)
Lithospermum erythrorhizon	Shikonin derivatives	(60) (61) (62)
Lycium chinense	Cerebroside	(63)
Morinda citrifolia	Anthraquinones	(64) (65)
Mucuna pruriens	L-DOPA	(66)
Nicotiana tabacum L.	Nicotine	(67)
Panax notoginseng	Ginsenosides	(68) (69)
Papaver somniferum	Morphine, Codeine, Sanguinarine	(70) (71)
Peganum harmala L.	β-Carboline alkaloids, Serotonin	(72)

Phytolacca americana	Betacyanin	(73)
Picrasma quassioides Bennett	Quassin	(74) (75)
Podophyllum hexandrum royle	Podophyllotoxin	(76) (77)
Polygonum hydropiper	Flavanoids	(78)
Rauwolfia serpentina Benth.	Reserpine	(79)
Salvia fruticosa	Rosmarinic acid	(80)
Orthosiphon Stamineus Benth.	Rosmarinic acid	(81)
Salvia miltiorrhiza	Cryptotanshinone	(82)
Sapium sebiferum	Tannin	(83)
Solanum chrysotrichum (Schldl.)	Spirostanol saponin	(84)
Solanum laciniatum Ait	Solasodine	(85)
Solanum paludosum	Solamargine	(86)
Stizolobium hassjoo	L-DOPA	(87)
Tabernaemontana divaricate	Alkaloids	(88)
Taxus spp.	Taxol	(89)
Taxus media	Paclitaxel, Baccatin III	(90)
Taxus cuspidata	Taxoids	(91)
Thalictrum minus	Berberine	(92) (93)
Thalictrum rugosum	Berberine	(94)
Torreya nucifera var. *radicans*	Abietane Diterpenoids	(95)
Trigonella foenumgraecum	Saponins	(96)
Trypterigium wilfordii	Cytotoxic Diterpene Tripdiolide	(97)
S. ionantha	Cyanidin	(98)
Psoralea corylifolia	Daidzein (Isoflavones)	(99)
Vitis vinifera	Resveratrol	(100)
Vitis vinifera	Anthocyanin	(101)
Phyllanthus debilis Klein ex Willd	Hydrolysable Tannin	(102)

2.1.1. Industrial applications of secondary metabolites

PSMs play a main role as bioactive and nutraceutical compounds. Nutraceutical is a term combined with “nutritional” and “pharmaceutical” and it is referred to the compounds within the food that act as a medicament (103). On Earth, there are at least 20,000 plant species, and every year, roughly 1600 new plant compounds are found (104). Approximately, 2500 species have been screened for their pharmacological activity. In the USA, 25 % of drugs are obtained from plants. Terpenes, phenols, alkaloids, and cyanogenic glycosides are sources for cosmetics, pharmaceutics, food additives, flavor, agrochemical biopesticides, and natural pigments (12)(105). Alkaloids show chemoprotective, analgesic, anti-plasmodial, and antibacterial properties. Phenolic compounds hold biological properties such as ant apoptosis, antiaging, antioxidant, anticarcinogen, anti-inflammatory, cardiovascular protection, angiogenesis inhibition, cell proliferation activity, and antiatherosclerosis (103). Some SMs and their industrial utilization are given in Table 2.2.

Table 2.2. Production of SMs from plants and their industrial uses (13) (106) (107) (108) (109) (110).

Secondary metabolite	**Type of plant species**	**Industry**	**Industrial application**
Codeine (alkaloid)	*Papaver sominiferum*		Analgesic, antitussive, antidiarrheal, antidepressant, sedative and hypnotic properties
Diosgenin (steroid)	*Dioscorea deltoidea*		Antifertility agent
Vanillin	*Vanilla spp.*		Vanilla
Diosgenin	*Dioscorea deltoidea*		Antifertility
Morphine	*Papaver somniferum*		Analgesic
Shikonin	*Lithospermum erythrorhizon*		Antibacterial
Anthraquinones	Morinda elliptica		Antimicrobial

Quinine (alkaloid)	*Cinchona ledgeriana*	Pharmacological activity	Antimalaria, antipyretic, analgesic, anti-inflammatory, antiarrhythmic, bacteriostatic
Digoxin (glycoside)	*Digitalis lanata*		Heart stimulant
Digitalis	*Digitalis purpurea*		Heart disorders treatment
Scopolamine	*Dhatura stramonium*		Anticholinergics (alkaloid)
Rosmarinic acid	*Coleus blumei*		Spice, antioxidants, anti-inflammatory and antimicrobial activities
Vincristine and Vinblastine	*Catharanthus roseus*		Antileukaemic
Taxol	*Taxus brevifolia*		Anticancer
Resperine	*Rauwolfia serpentine*		Antihypertensive
Atropine	*Atropa belladonna*		Anticholinergic
Hyoscyamine	*Hyoscyamus niger*		Anticholinergic
Berberine	*Thalictrum minus*		Antibacterial/viral, Antidiabetic, Anti-inflammatory
Catechins	*Camellia sinensis*		Antibacterial, antioxidant
Hydrolysable Tannins	*Phyllanthus debilis* Klein ex Willd		Antioxidant, Anticancer, and Antimicrobial
Triterpene	*Eriobotrya japonica*		Antiinflammation Antitumor, Ant diabetes
Ginseng saponins	*Panax ginseng*		Dietary supplement
Podophyllotoxin	*Podophyllum hexandrum*		Anthelminthic Antifungal, Antitumor
L-Dopa	*Mucuna pruriens*		A potent drug for Parkinson's disease
Camptothecin	*Camptotheca acuminate*		Antitumor
Phenolic compound	*Larrea divaricata*		Arthritis, Digestive disorders, Rheumatism, Venereal diseases
Pyrethrin	*Chrysanthemum cinerariaefolium*	Agrochemical	Insecticidal
Lutein	*Tagetes erecta*		Insecticidal, Antiherbivore

Nicotine	*Nicotiana rustica*		Insecticidal
Anthocyanin	*Vitis vinifera*		Antioxidative
Quinine (alkaloid)	*C. ledgeriana*	Food & drink	Bittering agent
Saffron	*Crocus sativa*		Flavoring/coloring agent
Capsaicin	*Capsicum frutescens*		A pungent food additive
Anthocyanin	*Euphorbia milii*	Dyes	Textile dyes
Carotenoids			Colorant
Jasmine	*Jasmium* sp.	Cosmetics	Perfume

2.2. *VITIS VINIFERA* (L.)

Grapevine (*Vitis vinifera* L.) is high in antioxidants, bioactive chemicals, and nutraceuticals, and it is one of the most beneficial fruits for human consumption (111). It is cultivated as a commercial fruit crop and has been developed and adapted to a wide diversity of climates owing to its long cultivation (112). Production and consumption of *Vitis vinifera* (L.) as fresh fruit and for making wine, juice, jam, jelly, raisins, and drugs has increased due to the high nutritious values for humans. Phenolic compounds are found in grapes at approximately 75%, present in the skin and seeds (113). Phenolic compounds express many roles as anti-inflammatory, anticancer, antifungal, antibacterial, and antioxidant activity. Plant phenolic compounds are divided into simple phenols (coumarins and phenolic acids), flavonoids, hydrolysable and condensed tannins, stilbenes, lignins, and lignans (Figure 2.2) (112) (114). Many studies have shown the production of different phenolic compounds using *V. vinifera* CSC such as anthocyanins, resveratrol, catechins, condensed tannins, and other derivatives of resveratrol (cis and trans- piceid) (115) (116) (117).

Vitis vinifera (L.) cv. Öküzgözü is a Turkish grape variety and using this grape, Turkish wine is produced. *Vitis vinifera* (L.) cv. Öküzgözü is the most cultivated grape variety in Turkey, a rounded dark red fruit (118).

2.3. *VITIS LABRUSCA* (L.)

Vitis labrusca (L.) is an American grape species. There are many varieties of *Vitis labrusca* (L.) such as Isabel, Niagara, Concord, and Bordo. Those grape varieties are developed in

Brazil, and processed for juice production, but also the table, wine production, grappa, sweets, and jams (119). *V. labrusca* cv. Isabel is the main variety followed by the Ives variety and both are utilized for usual wine production. Due to its high productivity and its easy adaptation to different environmental conditions, the "Isabel" cultivar is highly fertile and very rustic, spread throughout Brazil (120). The cultivar 'Isabel' was obtained by a cross with an unknown *Vitis vinifera* susceptible to mildew and black rot and it was imported into Europe in the 19th century because of its high resistance to grape phylloxera (121).

To the best of our knowledge, no study was found on the optimization of suspension culture, scale-up studies, and t-resveratrol production using the *Vitis labrusca* (L.) cv. Isabel (120) (121).

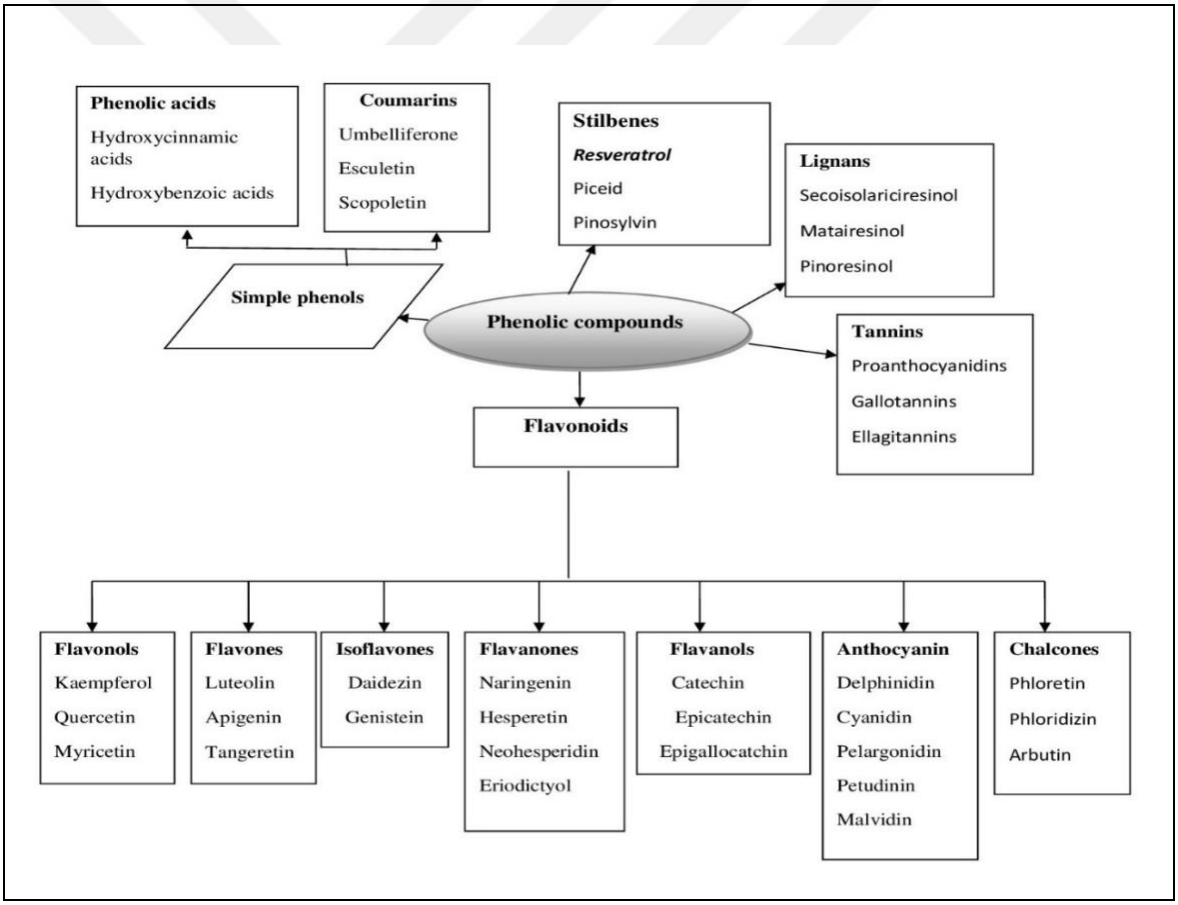


Figure 2.2. Classification of phenolic compounds (114)

2.4. RESVERATROL

Resveratrol is a phytoalexin of belonged to the stilbenes family which is isolated by the defense response of plants to abiotic and biotic stresses (122) (123). Resveratrol was found approximately in 72 plant species, but mainly it is produced by peanuts and *Vitis vinifera* (L.) (124). Resveratrol (3,5, 4- trans-hydroxy stilbene) is a white powder, with a low molecular weight of 228 g/mol and melting point around 253-255 ^{o}C (125) (126) (127) (128) (129). Firstly, t-resveratrol was synthesized in *Veratrum grandiflorum O. Loes* (white hellebore) and *Polygonum cuspidatum* syn. *Fallopia japonica* (knotweed) (130).

There are different forms of resveratrol such as glycosylated form (piceid) or free (aglycone) and its oxidative dimerization. Because of the two phenol rings that are connected by a styrene double bond, resveratrol can be found in both cis- and trans-isomeric forms. However, t-resveratrol can be converted to cis-resveratrol by UV photoisomerization. (Figure 2.3) (2). The maximum absorbance for cis-resveratrol is 286 nm, whereas for t-resveratrol it is 306 nm. Isomers of resveratrol (cis and trans) are very sensitive to light and their protection from light lead to stable t-resveratrol in buffers with pH ranges from 1-7 around 28 days whereas cis-resveratrol is broken down at pH= 10 (128).

Hundreds of studies have shown the role of resveratrol as an antioxidant, antifungal, antibacterial, cardioprotective, antitumor, and neuroprotective. Furthermore, in organisms such as yeast and metazoans and higher organisms, resveratrol increases lifespan through the sirtuin proteins activation (3) (124) (129) (131) (132) (133) (134) (135) (136).

The common strategy for stilbenes and specifically for t-resveratrol production is to use conventional plant extraction procedures. In some cases, the t- resveratrol amount is often low in amount for a plant species and it might be activated only under specific climate or stress, during a specific developmental stage, or when nutrients are available. Due to the *in vitro* cell, population homogeneity, the abundance of material, good reproducibility of circumstances, and the rapid cell growth rate, the use of *in vitro* culture is one suitable way for t-resveratrol production. The main advantage of PCSC is the production of t-resveratrol in response to abiotic and biotic stresses without the need for using genetic modifications (130).

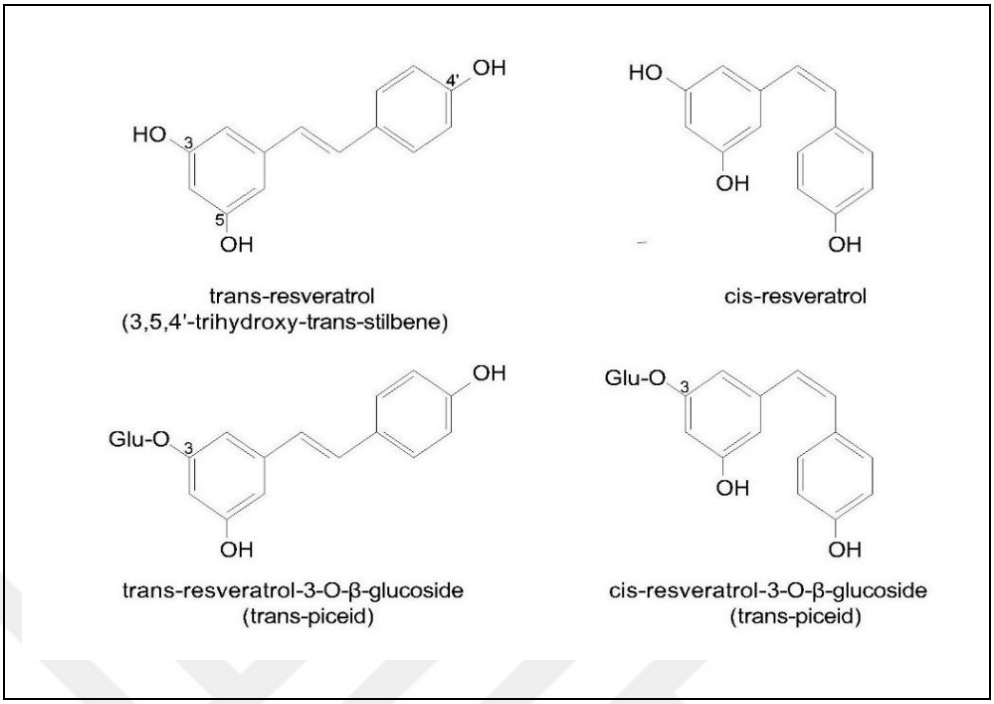


Figure 2.3. Chemical structures of t-resveratrol and its derivatives (128)

2.4.1. t-Resveratrol biosynthetic pathway

Resveratrol is formed from amino acids (tyrosine or phenylalanine) through the phenylalanine/polymalonate path (Figure 2.4). As a byproduct of the shikimate pathway, phenylalanine serves as a key intermediate between the primary metabolism and secondary metabolism of flavonoids, phenylpropanoid, and stilbenes. Firstly, the enzyme phenylalanine ammonia-lyase (PAL) catalyzes the conversion of phenylalanine into cinnamic acid. Para hydroxycinnamic acid is produced by phenylalanine and tyrosine as precursors. The obtained cinnamic acid is then hydroxylated to a p-coumaroyl-CoA by a 4-coumarate CoA ligase (4CL) and cinnamate-4-hydroxylase (C4H). Then, by the action of stilbene synthase (STS), one molecule of p-coumaroyl-CoA is condensed with three malonyl CoA units to produce t-resveratrol (122) (128) (135) (137) (138) (139).

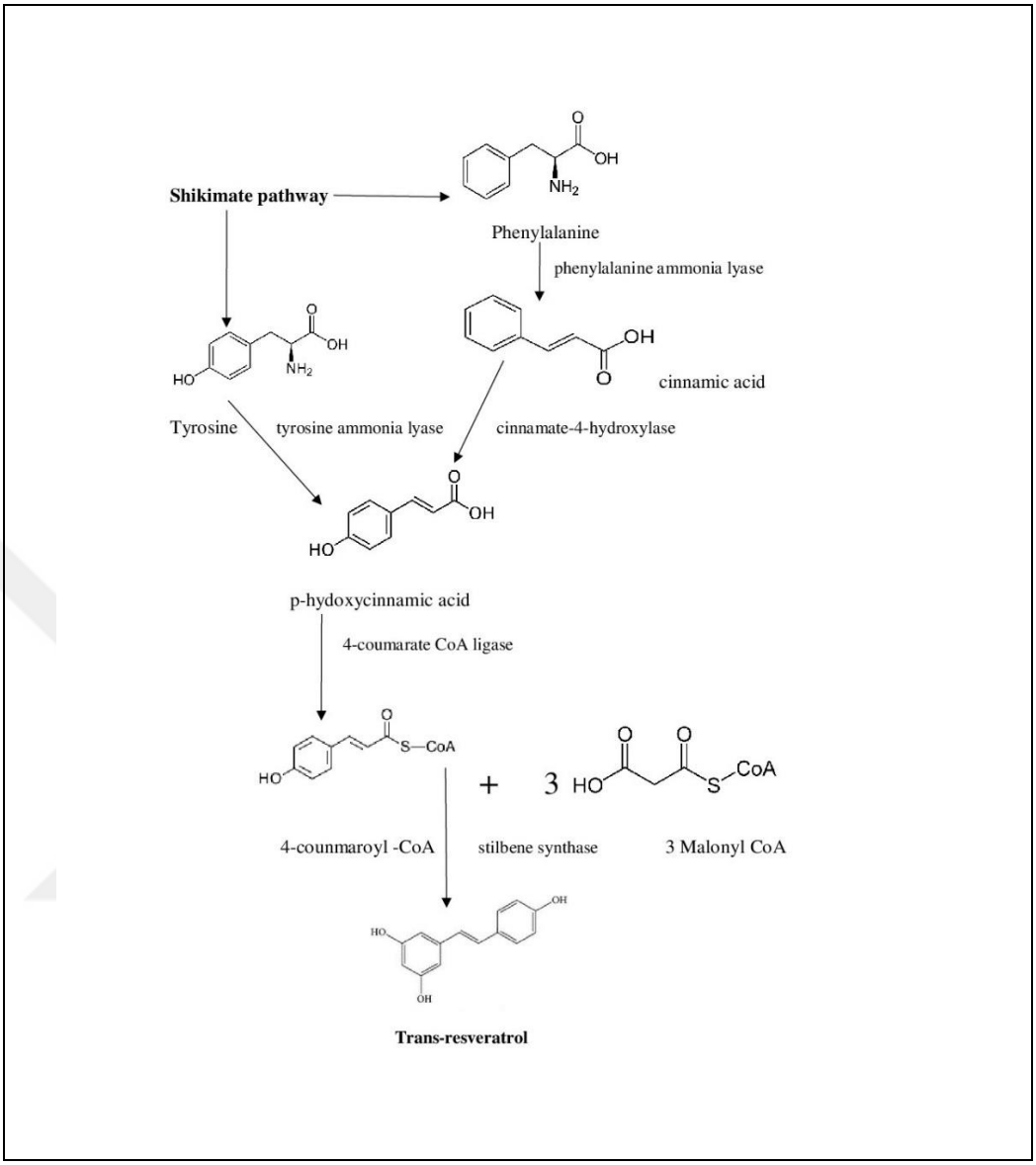


Figure 2.4. Biosynthesis pathway of t-resveratrol (128) (130)

2.5. PLANT CELL CULTURE TYPES USED FOR SECONDARY METABOLITES PRODUCTION

Plant cell and tissue culture technologies formed from explants, such as stems, roots, plant leaves, and meristems are used for both micropropagation and SMs extraction (4). There are five main types of PTC used for SMs production as shown in Table 2.3.

Table 2.3. Characteristics of PCSC and organized culture (root, shoot, and embryo) (140).

Characteristics	**Suspension**	**Untransformed root culture, shoot, embryo**	**Hairy roots**
Size	10-20 μm in length, often aggregated, up to 2 mm in diameter	Large organized structures, up to 2 cm in length	Long, highly branched roots; lack of geotropism Genetically and biochemically very stable
Doubling time (t_d)	2-7 days	5-15 days	1-8 days
Aeration requirement	Low Gas mixtures containing ethylene or CO_2 may be important for cell metabolism	Low Require certain levels of CO_2 or ethylene	Low
Shear stress sensitivity	Partial	Probably, sensitive	Sensitive

2.5.1. Plant callus and cell suspension culture

Undifferentiated cells, including callus and CSC, were long believed to be incapable of advancing the production of SMs. The ability of undifferentiated cells to produce SMs has just recently been demonstrated in the history of *in vitro* methods. Plant cell line selection is crucial for SM production and it is carried out using small aggregate or single cells from the PCC mixed population. To establish a PCSC, a sterile explant of plant tissue is first placed on a solid medium with the necessary nutrients and hormones optimized for the induction of the callus (141). PCSC holds many advantages such as manipulation of environmental conditions, free of soil conditions or climatic changes, less differentiated cell populations, avoidance of harmful biological impacts (microorganisms and insects) that affect the maintenance of the production system, ability to choose cultivars with higher plant cell production, automation control in cell development, and regulation of the metabolic process. Further, PCSC is distributed homogeneously throughout the culture fluid which allows the off-line measurement of parameters such as the biomass concentration. Homogeneous

distribution enhances the uptake of nutrients in submerged culture. High cell density (> 50g/L) and high yield of SMs can be reached using PCSC. It is possible to cultivate cell suspensions in large volumes (*Nicotiana tabacum* 20,000L stirred reactor) (Figure 2.5). Using PCSC, mass propagation of cells and better control for scale-up can be achieved. Important process parameters including T, pO_2, pH, and substrate concentration can be controlled due to the not limited mass transfer. Due to their features, PCSC is a suitable and preferable material for the investigation of SMs using bioreactors (8) (10) (11) (12) (13) (14) (15)(142) (143) (144).

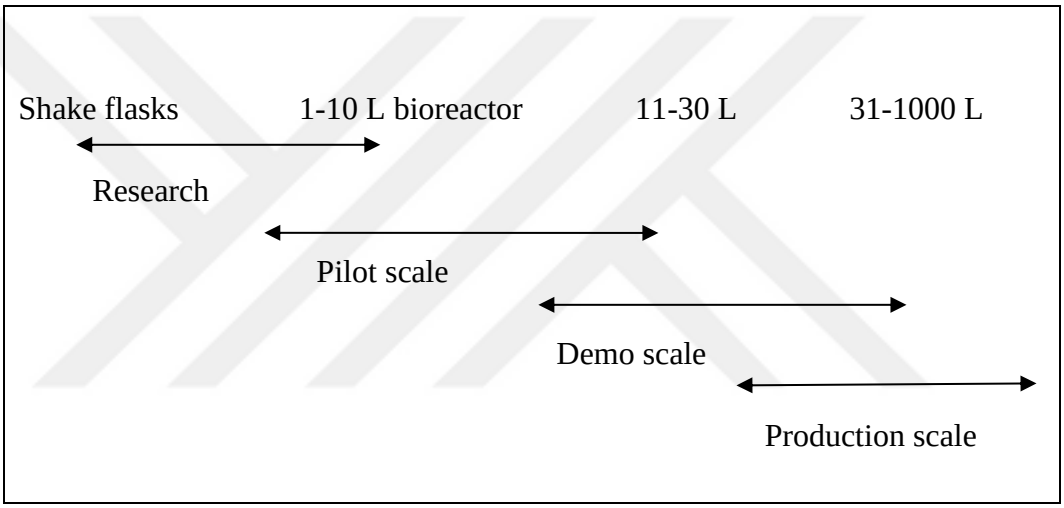


Figure 2.5. Scale-up cultivation of plant cell and tissue culture in a bioreactor (144)

2.5.2. Organ culture

Organogenesis is the development of unipolar organs. Cell division occurs in unorganized cell masses with callus development or in organized cell masses with the direct production of meristematic centers in the explant tissues. Meristematic centers formed directly on the plant explant can be developed into shoots, roots, or somatic embryos (144). For the production of several phytochemicals, such as the morphinan alkaloids of *Papaver somiferum*, dimeric indole alkaloids of *Catharanthus roseus*, and tropane alkaloids of numerous Solanaceous species, the link between morphological and biochemical differentiation must be broken (145).

2.5.3. Shoot culture

Shoot culture are developed either from explants directly or from calli cultivated in a solid or liquid media. Shoot culture produced in aerial parts of plants has been considered suitable for SM. Shoot culture provides a rapid growth, high multiplication appropriate system to control the medium composition, automated inoculation, mechanical separation, and effective transportation to the plant's final growth and development stage. Production of many SMs have been reported by using shoot culture. For example, monoterpenoid essential oil flavors are not able to be produced from calli or cell suspensions culture because of a lack of oil-secretory tissues. Vinblastine and vincristine, catharanthine, and vindoline, precursors of the dimeric indole alkaloid as well have been known to be produced from shoot culture (144) (145).

2.5.4. Root culture

Hairy roots and untransformed roots are two types of root culture. Hairy root culture are achieved by transformation with *Agrobacterium rhizogenes* (146). Hairy root culture have been generally used for *in vitro* production of PSMs without losing biosynthetic or genetic stability (12) (142) (147). Advantages of hairy root culture and attention are given to those due to phenotype and genotype stability, fast growth, autotrophy in plant hormones, and high levels of SMs production. From hairy roots, tropane alkaloids (scopolamine), pharmaceutical substances, pesticides, flavorings, and dyes can be produced (145).

2.6. INCREASING OF SECONDARY METABOLITE PRODUCTION IN PLANT CELL SUSPENSION CULTURE THROUGH MANY STRATEGIES

The limitation of PTC technology is the production of SMs in low yield due to the failure of cell culture to gather higher amounts of SMs compared to organ culture or whole plants.
Many factors may restrict SMs production from PCs such as metabolic inhibition phenomena, degradation of the product, compound's biochemical instability in the presence of producing cells, and loss of compounds due to their volatile feature (148). This could be improved by using different strategies such as screening of high-yielding cell lines, medium

optimization, immobilization, precursor feeding, biotransformation, elicitation, and scale-up of PCs in the bioreactor (149) (150) (151).

Strategies to increase SMs production should incorporate both the plant biology perspective in selecting the host as well as the bioprocess engineer in optimizing/adjusting the process conditions. Typically, such a strategy involves two stages: in the first stage, the focus will be on the selection of cell lines as well as optimization of medium components, involving macro/microelements, carbon source type, and level as well as growth regulators. Lastly, the culture parameters, e.g. inoculum density as well as process parameters (pH, T, aeration rate, agitation speed) need to be optimized. Mainly the objective here is to produce enough biomass to later produce an SM of interest. The second stage focuses, in turn, on elicitation to induce SMs production and has the following tools at the disposition: permeabilization to better feed substrates or extract products, immobilization for the flow-through system, selective adsorption of a metabolite of interest, the use of two phase-system (biomass production – separate metabolite production) (150) (152).

2.6.1. Culture conditions and culture environment optimization

Selection of the plant with appropriate physiological, genetic, and biochemical features is the prior step that should be considered during PSMs production. After the target variety selection, the following step is the adequate explants selection for callus initiation. The obtained callus is subcultured many times to reach stable cell lines (153).

Generally, the PTC medium includes all the compounds essential for the development and growth of PCs and SMs production. The basic medium used for plant cell cultivation is MS, B5, LS, and Nitsch and Nitsch medium. For the establishment of PCSC, the selection of a suitable medium is crucial and is related to the plant type (142) (154). PTC medium is mainly composed of macro-micro nutrients (oxygen nitrogen, hydrogen (macro), iron, zinc (micro), vitamins (thiamin (B1), nicotinic acid, and pyridoxine (B6)), amino acids (L-glutamine, L-asparagine), carbon source (sucrose, lactose, galactose), plant growth regulators (gibberellins, auxins, cytokinins,), and some solidifying agents (agar, agarose, and gellan gum) for solid medium according on the type of plant, growth characteristics, and cultivation purposes (10) (14) (109) (142) (143) (150) (152)(154) (155) (156) (157).

Inoculum density (g/L) is an important parameter for the success of PCC on growth biomass and SMs production by affecting the biological environment of culture because of interactions between cells and between cells and the culture media. Plant suspension culture are initiated by inoculating friable callus (10 % packed cell volume (PCV) or 50-100 g/L) in the liquid medium, but it depends on plant species. Cell or organ growth often fails below a critical minimum inoculum density of 5% PCV. Understanding the inoculum and substrate requirement for cell growth development, plant culture optimization, and scale-up is necessary. In large-scale industrial culture, the impact of inoculum density on cell growth has been noticed (150) (158)(159)(160)(161)(162) (163).

PCSC grows at a temperature between 15°C and 35°C. At a temperature higher than 35 °C the growth rate decreases, and cell damage occurs whereas, at a temperature lower than 20 °C, cell growth is inhibited (22) (145).

Light is a physical factor that shows a basic role in morphogenesis and photosynthesis that change plant structural development. The direct connection of light with the content of chlorophyll and its fluctuation has led to the enhancement of many SM products (150) (164). The effect of light on SMs production varies between plants. For example, *Thermopsis lupinoides* produced a lupine alkaloid only using a green callus. Light is often essential for anthocyanin production. In contrast, nicotine production in tobacco cells and shikonin from *Lithospermum erythrorhizon* cell culture was stopped by the presence of light. The connection between the distance of a light source and its intensity is very important in the bioreactor to stimulate the production of SM. In PTC, fluorescent lamps are the main source of light (145) (165).

pH is also important because it affects plant growth and the activity of plant growth regulators. For PCSC, pH ranges from 5-6. Extreme pH should be avoided (145) (166) (167).

2.6.2. Specialized techniques influencing secondary metabolites production in plant cells

2.6.2.1. Elicitation

Elicitation is a technique used to improve the quantity of SMs. Elicitors are compounds of biological origin that when introduced to a living cell system in small concentration, improve the biosynthesis of the SMs (166) (Figure 2.6). PCs' response to the elicitors is similar to the

defense responses to environmental factors or pathogen infections. The response occurs by activating an array of mechanisms, starting with the recognition of microbe-associated molecular (MAMPs) or pathogen-associated patterns (PAMPs) (168). When challenged by the elicitors, signal perception shown by PCs is receiving of elicitor by a receptor, fluxes in Ca^{2+} and other ions, rapid change in protein phosphorylation, activation of some proteins (mitogen-activated protein kinase (MAPK), G-protein, protein kinase, NADPH oxidase, and phospholipases). Cytoplasm acidifies as a result of H^{+}-ATPase inactivation, as the membrane polarization decreases, and extracellular pH increases. Production of ROS such as H_2O_2 and the superoxide anion, early and late defense gene expression, jasmonate production, and SM accumulation occur. The plant's resistance against future pathogen attacks can be increased by using elicitors (135) (168) (169) (170).

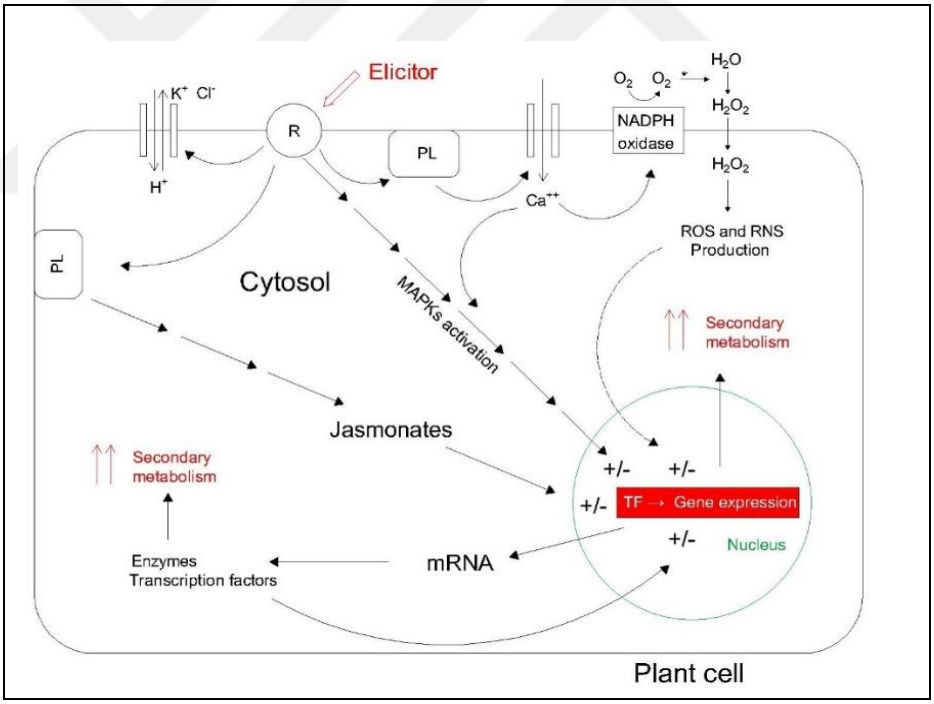


Figure 2.6. Elicitation mechanisms. Receptor (R), phospholipase (PL), mitogen-activated protein kinases (MAPKs), reactive oxygen species (ROS), reactive nitrogen species (RNS), and transcription factors (TF) (171)

Classification of elicitors

Elicitors are divided based on their nature (biotic and abiotic elicitors) and based on their origin (endogenous and exogenous elicitors) (Figures 2.7 and 2.8). Biotic elicitors include those derived from fungal, bacterial yeast and polysaccharides (cellulose, CHI, xanthan), whereas abiotic elicitors are non-biological substances divided mainly into physical, hormonal, and chemical elicitors (3)(12) (115) (122) (124) (125) (126)(133)(171) (172)(173) (174) (175) (176) (177).

Biotic elicitors have been shown to affect the synthesis of SMs in medicinal plants. The level of ginseng saponin in the Panax ginseng CSC increased as a result of oligo galacturonic acid presence. CHI improves stilbenes production, and total phenolic, and terpenic compounds. CHI stimulated the t-resveratrol production in the *V. vinifera* cv Barbera CSC (178). The amount of cryptoanshinone and tanshinone IIA produced in *Perovskia abrotanoides* root culture has been influenced by yeast extract. (179). Biosynthesis of rosmarinic acid and phenolic compounds were shown to be induced in *Salvia. miltiorrhiza* hairy roots (180). Applying endophytic fungal elicitor from *Fusarium sp*. E5 in the suspension culture of *Euphorbia pekinensis* resulted in the euphol and isoeuphpekinensin accumulation (181). The bacterial elicitor showed an effect on total ginseng saponin content in *Panax ginseng* hairy root culture. A fungal elicitor caused 2,5-dimethoxy-1,4-benzoquinone accumulation in a CSC of *Panax ginseng* (182). *Aspergillus niger* used as an elicitor in *A. Paniculata* (Burm.F.) Nees. CSC enhanced the accumulation of total flavonoids under *in vitro* conditions (183). *Hypericum perforatum* CSC were treated with fungal elicitors including *Botrytis cinerea, Fusarium oxysporum,* and *Phoma exigua,* which showed a rapid stimulation of SM (phenylpropanoid and naphtodianthrone contents) (184).

Physical elicitors are composed of light, osmotic stress, heat shock, salinity, wound stress, high hydrostatic pressure (HP), and drought (174)(175)(185). The effect of light was reported on gingerol and zingiberene production from *Z. officinale*. The effect of light was observed also on *Digitalis purpurea* L. (digitoxin production), and *Catharanthus roseus* (Vincristine and vinblastine production). The production of flavonoids and stilbenes has been stimulated by ultraviolet C (UV-C) in grape calli, leaves, and berries of various genotypes of *Vitis vinifera*. Light affected the anthocyanin production in *V. vinifera* cell culture (175) (186). The osmotic stress effect on hypericin and hyperforin production was observed in *Hypericum perforatum* plants. Under salinity exposure, alkaloid vincristine

production was observed in *C.roseus*, and glycine betaine production in *Triticum aestivum*. High hydrostatic pressure (HP) induces stress in the PCs reported also on *Glycine max* which increased isoflavonoid biosynthesis (174). Anthraquinones level was increased on *Morinda citrofilia* in presence of a high hydrostatic pressure elicitor. Synthesis and accumulation of SMs were noticed on plants that were exposed to drought stress (in roots of *Glycyrrhiza uralensis* was shown the effect of drought in glycyrrhizic acid content). Thermal stress increased the production of SM in the herb *Panax quinquefolius*. Production of ginsenoside in the hairy root culture of *P. ginseng* was influenced by light and temperature presence.

Chemical elicitors include metal ions or salts. Salts include $HgCl_2$, $MgSO_4$, $AgNO_3$, $VOSO_4$, $AlCl_3$, $NiSO_4$, $CoCl_2$, $CaCl_2$, $CdCl_2$, $CdSO_4$, $CuCl_2$, Zn ions, and KCl (171). Recently, the effect of heavy metal ions was studied on PSM production. The effect of Ag, Cd, Cu, and Ni ions was noticed in tanshinones production from *Salvia miltiorrhiza* hairy root culture. Heavy metal elicitors including cobalt, silver, and cadmium ions showed an effect on phenolic acid and anthocyanin production in *Viti's vinifera* CSC (132). The highest accumulations of shikonin and digitalin were observed in culture treated with cadmium and copper ions. The effect of copper ions was also observed on betalains production in *Beta vulgaris* and on betacyanins production from *Amaranthus caudatus* callus. The impact of cobalt, silver, and cadmium ions on cell viability and phenolic (3-O-glucosyl-resveratrol accumulation was observed in *Viti's vinifera* CSC (187) (188).

Hormonal elicitors include SA, GA, JA, and its derivatives (MeJA, MeSA, MeCD). GA, SA, and JA are known as effective elicitors for the production of rosmarinic acid, phenolic compounds, and alkaloids. JA was reported to induce anthocyanin production in *Vitis vinifera* and rosmarinic acid production in *Mentha piperita*. JA and MeJA have been used as elicitors for stilbene production in *Vitis vinifera* cell culture. SA has shown an effect on alkaloid and terpenoid production.

Exogeneous elicitors derived outside the cell include peptides, polysaccharides, enzymes, and fatty acids, whilst endogenous elicitors originating inside the cell include hepta-β-glucosides or galacturonide (187) (188). CHI is a structural compound of the various plant fungal pathogens' cell walls. The effect of CHI has been studied in plant systems like *Eschscholzia californica* and *Nicotiana tabacum* for secondary metabolite production (183). The presence of exogenous elicitors stopped the growth of *Catharanthus roseus* cell culture, but they stimulated the production of 5'-phosphodiesterase (PDase) (189). According to a

proteomic analysis of *Vitis Vinifera* Barbera CSC, 73 proteins consistently changed in samples treated with CHI compared with controls. CHI induced stilbene synthase protein and intracellular t-resveratrol accumulation and inhibited extracellular t-resveratrol accumulation. CHI has not affected cis-resveratrol production. There was no shown production of cis- and t-resveratroloside and cis- and t-piceid (total resveratrol mono-glucosides) (178). Adding 150 mg/L CHI to *Artemisia annua* L hairy roots, artemisinin accumulation increased 6-fold (1.8 µg/mg dry weight) (190). IS, MCoA, In-Ile, and Lin-Gln stimulated the synthesis of phenylpropanoids on *V. vinifera* cv. Muscat de Frontignan. Treatment with MCoA, In-Ile, Lin-Gln, or IS did not inhibit the growth of *V. vinifera* cv. Muscat de Frontignan cells. On the other hand, the biosynthesis and higher yields of phenolic compounds were improved by using biological substances such as MCoA, In-Ile, and IS (191).

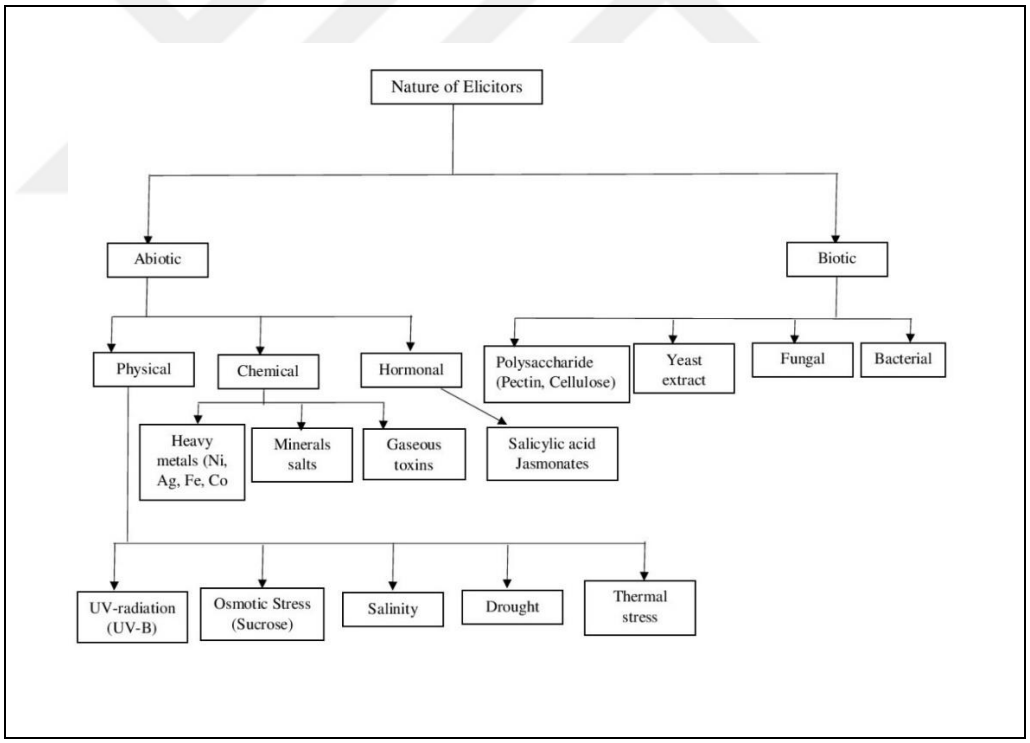


Figure 2.7. Elicitor classification depended on their nature (187) (188)

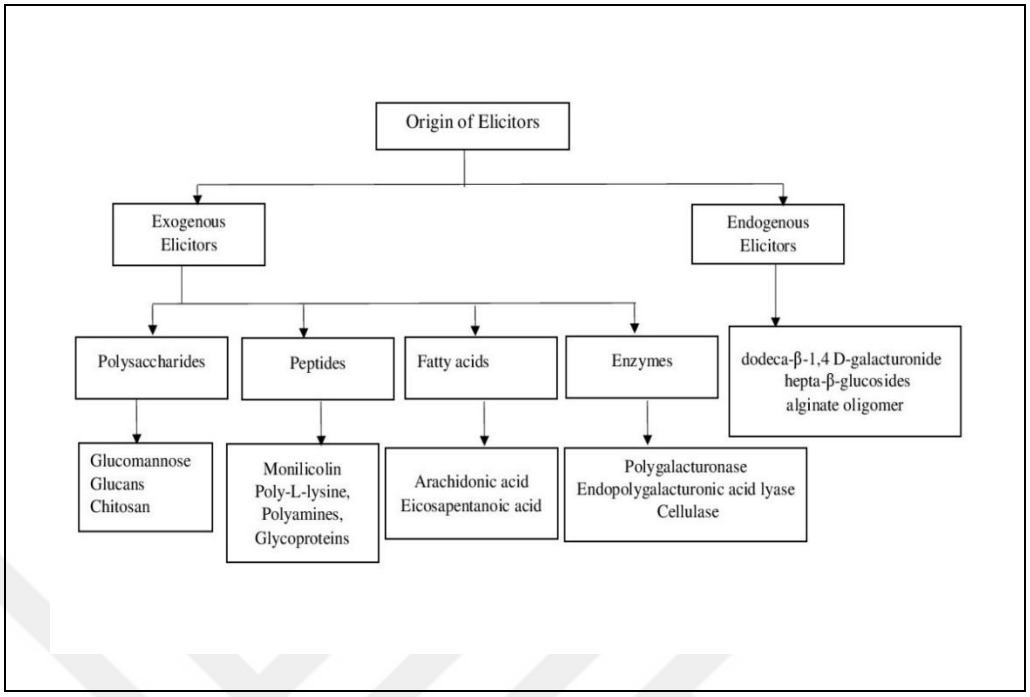


Figure 2.8. Elicitor classification depended on their origin (192)

Elicitors used for resveratrol production

There are many elicitors used for stilbene production referred to Table 2.4.

Most of the research has been focused on resveratrol and its derivatives *in vitro* production enhancement. by adding different elicitors such as MeJA, JA, SA, sucrose, β-cyclodextrins, HYPROB, DIMEB (192) (2) (3) (100) (122) (124) (126) (130) (133) (136) (137) (169) (176) (186)(193) (194) (195) (196)(197)(198) (199) (200) (201) (202) (203). Plant hormones including MeJA, JA, and its ester play important roles in the growth, development, and plant defense against pathogen attack. MeJA is one of the major elicitors applied in grapevine CSC to produce resveratrol. Stilbene synthase, a crucial enzyme in the production of resveratrol, was found to have increased expression and activity when cells were treated with MeJA (204). In *Vitis vinifera* CSC, sucrose combined with CDs and MeJA up-regulated the expression of defense genes and increased the stilbenes amount (126). The *Vitis vinifera* variety, culture conditions, and elicitors all have a significant impact on the amount of t-resveratrol that can be produced as illustrated in Table 2.4.

Based on many references (Table 2.4) resveratrol and its derivatives are not easily soluble in aqueous solutions and are subject to aggregation and oxidation phenomena that result in the production of true micelles. Because of that, using cyclodextrins protects extracellular

resveratrol by complex formation. This results in a significant increase in resveratrol production.

The results showed a higher amount of t-resveratrol obtained by adding Cavasol (4963 mg/L t-resveratrol) followed by DIMEB (4680 mg/L t-resveratrol) and HYPROB (4110 mg/L t-resveratrol) in *V. vinifera* cv. albino Monastrell CSC (205). Using DIMEB as an elicitor in the same concentration (50 mM), greater production of t-resveratrol is seen in the *V. vinifera* cv. albino Monastrell CSC ((4680 mg/L t-resveratrol) (205) than in *V. riparia V. berlandieri* (911 mg/L t-resveratrol), *V. amurensis* (225 mg/L t-resveratrol), *V. vinifera* cv. Merzling (4 mg/L t-resveratrol), and in *V. vinifera* cv.s Pinotnoir (0.5 mg/L t-resveratrol) CSC (176). A combination of 100 µM MeJA + 50 mM Methylated CDs led to a higher production of extracellular resveratrol than each elicitor used alone in *V. vinifera* cv. Gamay (3100 mg/L t-resveratrol) (199), because they act synergistically, including the expression of steroid sulfatase and the phenylpropanoid path resulting in a significant enhancement in the level of resveratrol. MeJA and CDs do not affect cell growth or cell viability and are very valuable elicitors for resveratrol production using plant cell suspension. Many results performed using SA + MeJA and adsorbent (HP2MGL) give a high amount of t-resveratrol (100). CDs + ethylene; and CDs+MeJA+ethylene have given higher resveratrol than CDs (CAVASOL) + SA or CDs (CAVASOL) + MeJA + SA in *V. vinifera* cv. Monastrell (133) (195). The results showed that $CdSO_4$, sucrose, and MeJA are effective elicitors in the CSC of *V. vinifera* cv. Öküzgözü, Gamay, Kalecik karası varieties (177). Grapevine CSC produced derivatives of t-resveratrol when treated with MeJA at different concentrations (3) (196) (203). In the article (117), maximal production of polyphenols was achieved using media with a high amount of minerals and high sucrose. The CSC of *V. vinifera* cultivated in an STR showed similar characteristics of growth and SMs production that were found in Erlenmeyer. *V. vinifera* cells produced 1200 mg/L of anthocyanins, 220 mg/L of proanthocyanidins, 8 mg/L of catechins, and 30 mg/L trans-priced in the media without elicitors (116). MeJA increased phytoalexin accumulation in *V. vinifera* cv. Gamay Freaux var. Teinturier cells grown in vitro. MeJA improved the accumulation of intracellular stilbenes, primarily (Z)- and (E)-piceid (3,5,4′-trihydroxystilbene-3-O-ß-glucoside), when it was introduced at the beginning of the exponential development phase (3). Treatment of cells with MeJA in combination with sucrose was adequate in the production of both anthocyanins and piceids in cells, and the production of extracellular t-resveratrol and piceids (196). Using 0.1 and 1 mM Na-orthovanadate was not helpful in t-resveratrol production whereas this elicitor promoted the

production of extracellular cis-resveratrol. MeJA was very effective in the accumulation of both intracellular and extracellular cis- and t-resveratrol. In control samples, cis-resveratrol was found in low amounts. JA showed less effect than MeJA for intracellular resveratrol accumulation, but it stimulated extracellular resveratrol accumulation, especially cis-resveratrol (202). JA and SA as elicitors and HP2MGL as adsorbent were added into the *V. vinifera* cv. Gamay Fréaux var. Teinturier CSC and the results showed that they successfully worked synergistically to enhance the production of t-resveratrol (2666.7mg/L) (100). After treatment with 10 M JA, 1 mg/mL GLU, and 200 g/L XAD-7, *V. vinifera* cv. Gamay Fréaux var. Teinturier cells produced resveratrol at a level of about 2400 mg/L (124). High t-resveratrol (200–315 mg/L) production by *V. amurensis* callus culture transformed with the rolB gene of *A. rhizogenes* was reported in the article (206). Dimethyl- β-cyclodextrins effect before and after inoculation with *Xylophilus ampelinus* on resveratrol production was studied in *V. vinifera* cv. Gamay CSC. The results showed that dimethyl-β-cyclodextrins to act as elicitors on t-resveratrol production do not need the co-cultivation with *Xylophilus ampelinus*. Dimethyl-β-cyclodextrins maintain a high level of peroxidase activity and protect grapevine cell suspensions against bacteria (207).

Table 2.4. Bioproduction of t-resveratrol and its derivatives from *Vitis vinifera* (L.) varieties using different elicitors.

Type of *Vitis vinifera* variety	**Culture Type**	**Type of elicitors**	**Stilbenes production (mg/L)**	**Ref.**
Gamay Freaux var. Teinturier	Erlenmeyer	No elicitor	Stilbene glucosides (150 mg/L)	(113)
Gamay Freaux var. Teinturier	Erlenmeyer Bioreactor	No elicitor	Anthocyanin (1200 mg/L) Piceids (30 mg/L) Proanthocyanidins (220 mg/L) Catechins (8 mg/L)	(116)
Gamay Freaux var. Teinturier	Erlenmeyer Bioreactor	No elicitor	Stilbenes (330 mg/L) Stilbenes (280 mg/L)	(194)
Gamay Freaux var. Teinturier	Erlenmeyer	25 µM MeJA	(Z), (E) – Piceids (200 mg/L)	(3)
Cabernet Sauvignon	Erlenmeyer	25 µM MeJA	(Z), (E) – Piceids (400 mg/L)	(203)
Gamay Freaux var. Teinturier	Erlenmeyer	10 µM MeJA	(Z), (E) – Piceids (840 mg/L)	(203)

Gamay Freaux var. Teinturier	Erlenmeyer	20 µM MeJA 20 µM MeJA + 20 g/L sucrose	Extracellular t-R and piceids (12 mg/L) Intracellular t-R and piceids (120 mg/L) Extracellular t-R and piceids (46 mg/L) Intracellular t-R and piceids (103 mg/L)	(196)
Barbera	Erlenmeyer	10 µM MeJA	Intracellular resveratrol (23.94 mg/L) Extracellular resveratrol (7.98 mg/L)	(202)
Roostock 41 B	Erlenmeyer and Bioreactor	200 µM MeJA	Resveratrol (150 mg/L) Resveratrol (209 mg/L)	(136)
Monastrell	Erlenmeyer	5 µM MeJA 100 µM MeJA	Resveratrol (798 mg/L) Resveratrol (3074 mg/L)	(133)
Cabernet Sauvignon	Erlenmeyer	UV-C + 100 µM MeJA	Intracellular resveratrol (2000 mg/L)	(186)
Gamay	Erlenmeyer	100 µM MeJA	Resveratrol (20 mg/L)	(199)
Italia	Erlenmeyer	25 µM MeJA 25 µM JA	Stilbenes (970 mg/L) Stilbenes (1023 mg/L)	(169)
Gamay Freaux var. Teinturier	Erlenmeyer	MeJA 10 µM + SA 500 mM + resin H2MGL	Resveratrol (2667 mg/L)	(100)
Gamay Freaux var. Teinturier	Erlenmeyer	10 µM JA + β-glucan+ Amberlite resin	Resveratrol (2400 mg/L)	(124)
Gamay	Erlenmeyer	5 mM DIMEB DIMEB+ Xylophilus ampelinus	t-R (148-184 mg/L) t-R (202.9 mg/L)	(207)

Monastrell albino	Erlenmeyer	50 mM DIMEB 50 mM HYPROB 50mM CAVASOL	t-R (4680 mg/L) t-R (4110 mg/L) t-R (4963 mg/L)	(205)
Monastrell albino	Erlenmeyer	50 mM DIMEB DIMEB and MeJA 50 mM DIMEB 50 mM HYPROB 50 mM G2βCD	t-R (753 mg/L) t-R (3651 mg/L) t-R (3400 mg/L) t-R (3000 mg/L) t-R (650 mg/L)	(137) (201)
Gamay Rouge	Erlenmeyer	50 mM DIMEB 50 mM HYPROB 50 mM G2βCD 50 mM RAMEB	t-R (3000 mg/L) t-R (990 mg/L) t-R (400 mg/L) t-R (3320 mg/L)	(201)
Gamay	Erlenmeyer	50mM Methylated CDs	Resveratrol (900 mg/L)	(199)
Monastrell	Erlenmeyer	50 mM Undefined CDs	Resveratrol (600 mg/L)	(133)
Berlandieri Riparia	Erlenmeyer	50 mM DIMEB	Resveratrol (911 mg/L)	(176)
Amurensis	Erlenmeyer	50 mM DIMEB	Resveratrol (225 mg/L)	(176)
Merzling	Erlenmeyer	50 mM DIMEB	Resveratrol (4 mg/L)	(176)
Pinot noir	Erlenmeyer	50 mM DIMEB	Extracellular resveratrol (0.5 mg/L)	(176)
Monastrell albino	Erlenmeyer	50 mM DIMEB + 100 µM MeJA 50 mM DIMEB alone	Resveratrol (365000 mg/L) Resveratrol (60000 mg/L)	(137)
Gamay	Flasks	50 mM Methylated CDs + 100 µM MeJA	t-R (3100 mg/L)	(199)

Monastrell	Erlenmeyer	50 mM Undefined CDs + 100 µM MeJA 50mM CAVASOL + 1 mM ethylene 50mM CAVASOL + 100 µM SA 50mM CAVASOL + 100 µM MeJA + 1 mM ethylene 50mM CAVASOL + 100 µM MeJA +100 µM SA	Resveratrol (3000 mg/L) t-R (680 mg/L) t-R (260 mg/L) t-R (1540 mg/L) t-R (1040 mg/L)	(133) (195)
Monastrell	Erlenmeyer	50 mM CD + 1 µM coronatine 50 mM CD + MeJA	Resveratrol (943 mg/L) Resveratrol (1600 mg/L)	(122)
Negramaro	Erlenmeyer	10 µM Coronatine MeJA	Resveratrol (35 mg/L) Viniferins (210 mg/L) Resveratrol (60 mg/L)	(126)
Rotundifolia	Erlenmeyer	100 µM MeJA	Extracellular resveratrol (< 23 mg/L) ε-viniferin extracellular (1.81 mg/L)	(2)

Amurensis Rupr.	Erlenmeyer	Phenylalanine	t-R (2.4 mg/L)	(206)
		SA	t-R (4.7 mg/L)	(206)
		MeJA	t-R (2.3 mg/L)	(206)
		Transformation with rol B gene	t-R (200–315 mg/L)	(206)
		Transformation with rol C gene	t-R (14.3 mg/L)	(208)
		5-azacytidine, block DNA methylation	t-R (5.1 mg/L)	(209)
Vitis labrusca L. cv. "Washington Concord"	Erlenmeyer	L-Alanine	t-R (2.2 mg/L)	(210)
Barbera	Erlenmeyer	MeJA Control Sucrose	t-R (0.11 mg/L) t-R (1.6 mg/L) t-R (6.7 mg/L)	(202) (178) (211)
Gamay Freaux var. Teinturier	Erlenmeyer	MeJA + sucrose	t-R (5.5 mg/L)	(196)
Kalecik Karası Öküzgözü	Erlenmeyer	1,5 mM $CdSO_4$ 0,2 M Sucrose 1,5 mM $CdSO_4$ 0.2 M Sucrose	t-R (3,65 mg/L) t-R (7,55 mg/L) t-R (2.96 mg/L) t-R (4.04 mg/L)	(177)

Öküzgözü Kalecik Karası	Erlenmeyer	MeJA 10 µM	t-R (11,681 mg/L) t-R (2.87 mg/L)	(177)
Öküzgözü Kalecik Karası	Erlenmeyer	Dark	t-R (9.48 mg/L t-R (3.1 mg/L)	(177)
Öküzgözü Kalecik Karası	Erlenmeyer	Light	t-R (4.35 mg/L) t-R (2.4 mg/L)	(177)

2.6.2.2. Permeabilization

In the vacuole, SMs are frequently stored. (150). When SMs are removed from vacuoles, permeabilization facilitates the process without affecting the cell viability and biosynthetic capacity of the cell culture. (141). Permeabilization can be reached by UV, electric pulse, pressure, sonication, heat, etc. Organic solvents as well have been used as permeabilizing agents such as isopropanol, dimethyl sulfoxide, and polysaccharides (CHI) (12).

2.6.2.3. Precursor feeding

Precursors are endogenous or exogenous compounds that can be transformed by the living system into SMs or useful compounds. The precursor increases the yield of the end product acting as an intermediate at the beginning or during the process. To produce indole alkaloids and tropane alkaloids in CSC, amino acids have been introduced as a precursor to alkaloid biosynthesis (166). For example, phenylalanine acts as a precursor of the N-benzoyl phenyl isoserine side-chain taxol; adding phenylalanine in *Taxus cuspidata* culture resulted in an increased yield of taxol (150).

2.6.2.4. Immobilization

Immobilized PCs are cell culture entrapped in agarose and calcium alginate or membranes. The most widely used matrixes are calcium alginate, agarose gelatin, carrageenan, and polyacrylamide (212). It's necessary to be considered many factors for being a good matrix such as they should be harmless to the cells, cheap and they should have good polymerization activity. Immobilized PCC shows better cell-to-cell contact and low shear stress. Elongated

viability of immobilized cells in the stationary phase provides the maintenance of biomass for an elongated period and increased product accumulation. Small bioreactors can reach high cell densities at lower costs and contamination risks. Downstream processing is simple using immobilized PCC (149)(150).

2.6.2.5. Biotransformation

Biotransformation includes the chemical transformation of supplied substances catalyzed by PCs, organ tissues, enzymes, or biological systems such as oxidoreduction, hydroxylation, glycosylation, hydrolysis, methylations, hydrogenation, esterification of different substrates, acetylations, and isomerization (12) (150).

2.6.2.6. Two-phase systems

The two-phase system is composed of an aqueous culture phase for cell development and a liquid or solid second phase to keep the SMs. The second phase consists of an additional site and its aim is the accumulation of secondary metabolite stored far from synthesizing cells or protoplasts, usually in the vacuole or specialized cells or structures (148). Two-phase culture systems increase secondary metabolite accumulation without having any effect on cell growth. Using of two-phase system effects:

a) Interference between cell growth and product accumulation is less, which allows expression of product at optimum production levels,

b) Avoidance of losing product resulting from the interaction between producing cells, environmental conditions, and release enzymes.

c) easy downstream process (212).

2.6.2.7. Two-stage systems

The two-stage are utilized when biomass growth is independent of the production phase. Parameters for biomass growth and product accumulation are optimized independently in a two-stage system. Firstly, cells are cultured in a nutrient-rich medium suitable for growth (stage I) then, cells are transferred to a medium for SMs production (stage II) (212) (213).

2.7. THE ROLE OF OPERATING CONDITIONS AND PLANT CELL CULTURE PROPERTIES ON BIOREACTOR PERFORMANCE

The features of PCSC including low oxygen requirement, sensitivity to shear stress, and slow growth rate make a complex way for the cultivation of PCs in the bioreactor (141) (214) (Table 2.5). These properties of PCs should rigorously be analyzed when bioreactors are used for scale-up cultivation of PCSC for SMs production (Table 2.6).

Unlike shoot, embryo, and root culture, PCSC are more suited for scale-up production based on their features and can be developed in bioreactors used for microbial and mammalian cell culture with small changes (plant bioreactor must have characterized by low shear stresses and efficient oxygen supply).

Table 2.5. Differentiation between microbial cells, PCSC, and mammalian cells (108) (215).

Features	**Microbial culture**	**PCSC**	**Mammalian cell culture**
Size	<1 μm in diameter	20-40 μm in diameter 100-200 μm in length	10-100 μm in diameter
Shape	Spherical, cylindrical	Spherical, cylindrical	Spherical
Single cells	Often	Aggregates 100 μm - 2 mm in diameter	Adherent (Sometimes)
Inoculum cell density	Low	50-100g/L or 10% PCV	High (5-10%)
Doubling time (t_d)	t_d=1-2h	t_d=2-7 day	t_d=20-50h
Sensitivity to shear stress	Insensitive	Sensitive	Highly sensitive
Stability of culture	Stable	Variable	Variable
Accumulation of product	Extracellular/Intracellular	Extracellular/ Intracellular (Vacuole)	Extracellular (Mostly)
Medium	Simple (Often)	Complex (Often)	Complex
Temperature	26-36 ^oC	17-25 ^oC	29-37 ^oC
Aeration	1-2	0.1-0.5	0.1

Foaming	High (Frequently)	Foaming (Sometimes)	Foaming (Sometimes)
pH for cell growth	3-8	5-6	7.0-7.4
PCV	Very high	High > 60 % PCV	Low middle
Scale up	Easy	Difficult	Difficult
OUR (mmol/l/h)	10–90	5-10	0.05-5
k **$_L$a (h^{-1})**	10–1000	15-50	0.25-10

Table 2.6. Implications considered during plant reactor design (141) (216).

Plant cell characteristics	**Factors to be considered for reactor design**
The respiration rate is low	OTR is required to be lower
Sensitive to shear stress	Low-shear conditions are required for operation (bubble-free aeration and impellers with low-to-shear stress)
Growth as aggregates	Cells as an aggregate have limitations in the mass transfer that limit the uptaken of nutrients
Aggregation is important for SMs	An optimal aggregate size may be needed for product formation by changing media compositions and environmental conditions.
Volatile organic substances (ethylene or CO_2) may be important for cell metabolism	Sparge gas mixtures containing the volatile substances are needed.
Product formation may be the non-growth associated product	A two-stage mode is required for maximal product formation.

2.7.1. Cell growth and Morphology

The diameter of PCSC ranges from 20- 40 µm and the length ranges from 100-200 µm, which makes PCs larger than microbial cells. Plant cell wall characterizes shape and cell size

and plays a dynamic role in cellular expansion and osmoregulation. The plant cell shapes range from spherical to nearly cylindrical shape. A significant part of the plant cell is occupied by a vacuole where SMs is mostly found. Plant cell growth is slow with a doubling time of about 2-7 (108) (217).

2.7.2. Aeration and agitation

PCs do aerobic respiration. However, due to their low growth rate, they request a lower amount of oxygen compared with microbial cells (150). The gas flow rate for PCSC is between 0.1-0.5 vvm. For PCs, maximum OUR is found between 5-10 mmol/ l/h whereas for microbial cells is 10-90 mmol//l/h. Maximum specific OTR for PCs is between 0.2 to 0.11 mmol/gdw/h. DO is mostly kept above 15-20 percent of air saturation which is found to be the critical oxygen level (Table 2.5). One of the major roles of the bioreactor is to facilitate the oxygen mass transfer from the gas phase to the liquid phase depending on the active cell's requirement. This can be achieved by optimizing parameters such as agitation speed, aeration level, gas mixing, impeller design, and selection of the bioreactor. Agitation is an important operating condition for oxygen transferring rapidly to the cells. The aeration effect on PCSC has focused on the effect of the k_LA in which agitation and aeration are related to each other. The k_LA for PCs is found between 15-50 h^{-1} (Table 2.5). High aeration causes foaming, which affects cell growth and the formation of products. Agitation's role is the distribution of cells, nutrients, and products throughout the liquid phase. Agitation is performed by sparging, impeller, or a combination of these two, to keep the uniform concentration of nutrients, pH, and gases in the bulk phase and increase the mass transfer rate. Agitation higher than 160 rpm (rotation per minute) is not suitable for PCSC (108) (141) (213)(218) (219).

2.7.3. Culture rheology

Rheological properties result from cell aggregate morphology and size, cell growth phase, biomass concentration, culture conditions, and cellular water content. When cell division is completed, PCs prefer to transfer to an elongated shape from a spherical shape causing high PCV at a given DW behaving as a non-Newtonian fluid during cultivation limiting mass and

heat transfer into PCs. Mixing, heat transfer, and aeration in the bioreactor are affected by rheological properties. Power-law fluid models involving pseudoplastic, Bingham plastic, and Casson fluid models have been utilized to represent the rheological features of high cell density in PCSC (108) (220)(221) (222).

2.7.4. Sensitivity to shear stress

PCs are more sensitive to shear stress compared to microorganisms, due to their great size, thin cellulose present in cell walls, and large vacuole which take 90-95 % of the cell's volume. High shear decreases cell viability and releases intracellular compounds, resulting in aggregation, and changes in the morphology and metabolism of cells (220). Sensitivity to shear stress varies amongst plant species and it depends on the cultivation regime and age of the culture. Impellers are the major origin of shear stress. Shear stress may also be produced by the gas sparging system, particularly when bubbles are produced through the bulk fluid and the suspension culture's surface. For instance, in *Nicotiana tabacum*, shear under 298/s (200 rpm) had no negative effect on cell viability whereas shear rates between 596-1193/s showed decreased cell viability (221). Critical shear stress for PCs has been reported between 50 and 200 N/m^2 (213).

2.7.5. Foaming and aggregation

PCs are characterized by growing as aggregates. Cell aggregate formation is mostly because of the cells' tendency to not divide after multiplication. The size of aggregate varies from >100µm up to <2 mm depending on the plant species and growth phase. Extracellular polysaccharides (ECP) secretion contributes to increased aggregation in the later phases of cell growth. Cell aggregations do not form stable suspensions and are undesirable because it complicates the bioreactor operation. The addition of higher cytokinin concentration, pectinases, and cellulase or decreasing calcium levels in the media composition may contribute to a decrease in the size of the aggregate (213) (219).

Foaming can be present on the culture surface due to the sugars, secreted proteins, presence of polysaccharides, sugars, secreted proteins, fatty acids, and air bubbles in the culture medium, that can block filters and tubes. Cells trapped in the foam occur due to oxygen and

nutrient deficiency outcoming in a reduction in the growth of cells and productivity and also producing a thick layer that adheres to the impeller shaft, sensors, and reactor wall preventing bulk mixing. The foaming can be minimized by decreasing agitation and aeration rate without affecting the mass transfer and mixing intensity by modifying actual impellers and establishing new impellers to perform better mixing at lower impeller velocity, constructing new aeration systems (such as gas basket, cage-aeration, and bubble-free aeration), using mechanical foam destruction such as ultrasonic irradiation, single or rotating plates, or addition of polypropylene and silicon-based antifoam agents (221) (223).

2.8. BIOREACTORS FOR PLANT CELL AND TISSUE CULTURE

The bioreactor is a major device of bioprocess in the industry. Bioreactors show the final step in the advancement of PCC-based bioprocessing. The bioreactor aims to ensure optimal conditions for cell functionality and metabolism by adjusting physical and chemical conditions (224). The design and operation mode of the bioreactor is subject to the type of plant and required conditions for the scale-up of desired product formation (225). The plant bioreactor contains a headspace volume, mixing system, aeration system, foam control, sensors for the control of pH, oxygen concentration, and temperature, sampling ports, a system for cleaning and sterilization, and a line for charging and emptying the reactor (225) (226). The bioreactor should be strong enough to cope with different treatments required such as exposure to pressure, high heat, and strong chemicals and washing and cleanings. The materials of the reactor should be carefully chosen to avoid the biological toxicity effects of the construction materials (Table 2.7). Polycarbonate and glass are the two major generally used materials for the rigid vessel (227). Different bioreactor designs are feasible to ensure the conditions for good cell growth and recombinant protein production (Figure 2.9) (225) (227) (228) (229).

Table 2.7. Effect of construction materials on the release of chemical compounds (227).

Construction Material	Chemical Compounds Released
PVC	
PVC – cement	Methylene, methyl ethyl ketone, chloroform, toluene, tetrahydrofuran, acetone, chloride, benzene, vinyl chloride, ethyl acetate, three organic Sn compounds, and cyclohexanone.
Polytetrafluorethylene (Teflon)	No compound released
Polypropylene or polyethylene	Phthalates and plasticizers
Epoxy materials	No compound released
Stainless steel	Ni, Cr, Mo, and Fe
Glass	B and Si

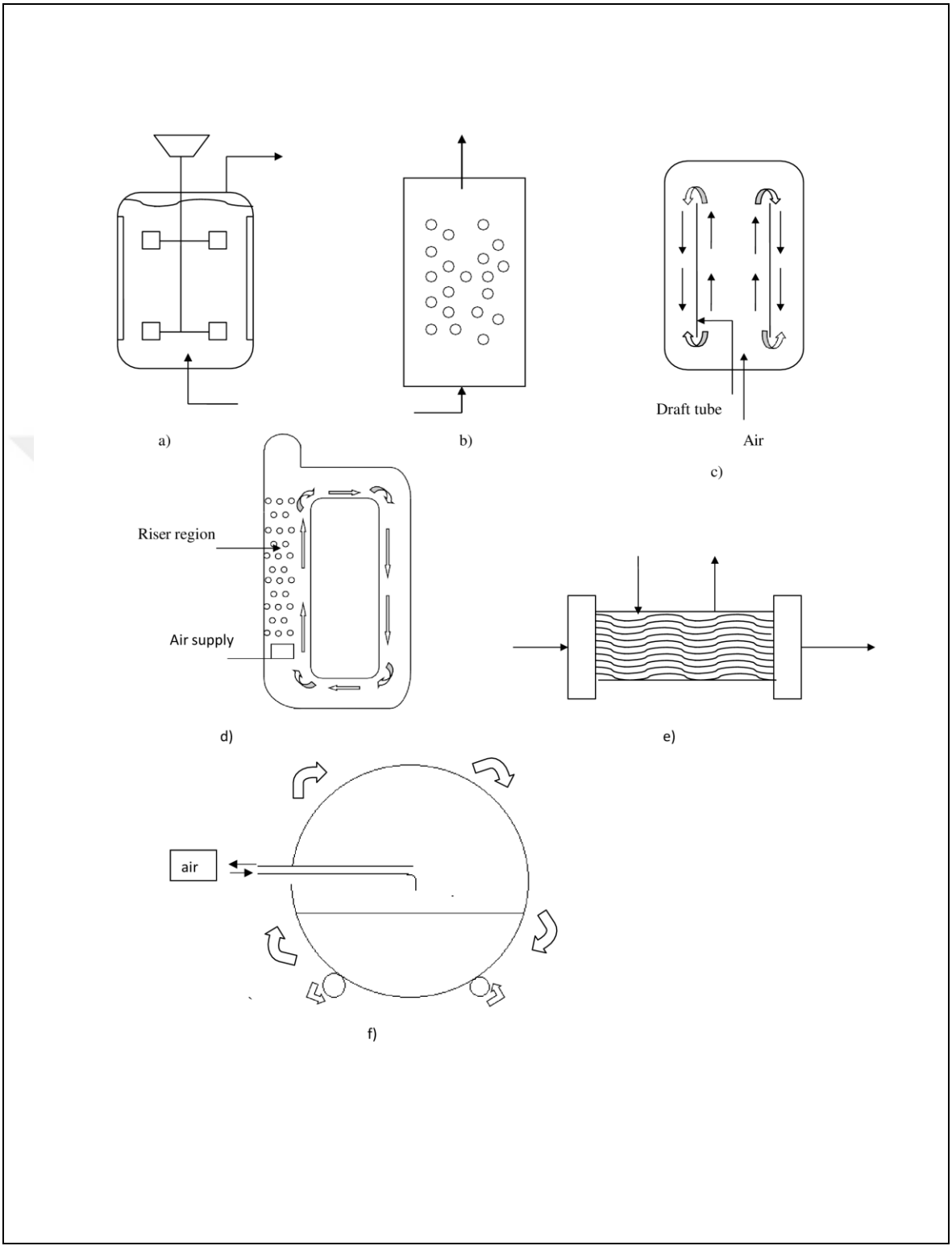


Figure 2.9. PCSC cultivation into different bioreactors. (a) STR, (b) Bubble column reactor, (c) Air-lift reactor (Draft tube), (d) Air-lift reactor (Outer loop), (e) Membrane reactor, and (f) RDR (141)

2.8.1. Characteristics of different types of plant bioreactors

Characteristics of PCs including slow growth rate, sensitivity to shear stress, and low oxygen lead to the consideration of many factors for the cultivation of PCSC in the bioreactor:

- Mixing is as important as aeration
- Optimal aeration with low stress to shear
- A slow growth rate provides long sterility of the process
- The cell aggregates can cause sampling problems, port blocking, and nonhomogeneous sample formation due to rapid settling, thus homogeneous and good mixing is necessary for efficient nutrient transport toward cells.

STR, airlift reactors, RDR, bubble columns, wave bioreactors, and membrane bioreactors are the most effective bioreactors for PCSC (Table 2.8). It is possible to cultivate cell suspension in large volumes (*Nicotiana tabacum* 20.000 L stirred reactor) (230). STR is favorable for plant cell suspension culturing at high cell density. They are dependent on the impeller and type of aeration system. The position of the sparger in the flow direction of the impeller provides temperature and mass homogeneity and optimal dispersion of gas. Many impellers have been designed for plant cell suspension to reduce the damage caused by hydrodynamic shear (Figure 2.10). Mostly, impeller systems displaying axial flow patterns with low velocity (up to 2.5 m/s) are considered adequate for PCC (231). The new model of impellers has been designed for shear-sensitive cell culture processes such as hydrofoil impellers, curved-blade disk turbines, cell-lift, and centrifugal impellers. Several impellers with low velocity including Prochem Maxflow, Intermig, and Scaba constructed for mammalian cell culture can be used for PCC (231). Pneumatically agitated bioreactors are characterized by uniform low-shear hydrodynamics characteristics. The advantage of air-lift loop and bubble column reactors is the low stress to shear offered. Unlike airlift reactors, bubble columns have no draft tube and mixing is achieved by the force of rising bubbles from the sparger. Airlift reactors consist of a draft tube, through which air moves between the vessel and the draft tube leading to the moving of medium throughout the reactor by the force of the air. Airlift reactors share low shear stress and high mixing characteristics. A RDR is another bioreactor that offers suspension homogeneity, low shear stress, and low cell growth around the wall. Novel bioreactors such as an external loop air-lift bioreactor, a

bubble-free loop fluidized bed bioreactor, and a centrifugal impeller bioreactor have also been designed to be used in the PCC scale-up. Fluidized-bed reactors are being suggested for industrial-scale use offering the chance of perfusion operation with a low-shear environment, ease for cell harvesting, and increased cell-cell interaction (224) (225) (231)(232). On the other hand, wave bioreactor shows the advantages of low shear environment and cost and time savings, low level of foaming, low risk of contamination, and easy operation. Heat and mass transfer are adjusted by rocking angle, rocking rate, and filling of medium volume. Studies have shown the utilization of wave bioreactors for tobacco, grape, and apple suspension cell cultivation (233). Membrane bioreactors are characterized by low shear stress, high cell density, and high volumetric productivity. However, large-scale operation in membrane bioreactors can result in operational issues such poor process stability, poor cell viability, product heterogeneity, membrane dirtying, and problems in mass and heat transfer (233).

Table 2.8. Characteristics of plant cell suspension culture bioreactors (234).

Reactor type	**Characteristics**	**Disadvantages**
STR	High OTR Good mixing of fluid and using of different impellers	Dead cells due to impeller High power consumption Difficult scale-up High shear stress
STR–low agitation and modified impeller	Medium OTR Low shear stress Uniform mixing	Deficient mixing at high cell densities Difficult scale-up
Bubble column	Medium OTR Low shear stress Low operational and capital cost Easy scale-up	Dead cells due to poor mixing. The large volume of air induces foaming. Growth of cells in the headspace. Less appropriate to high cell density culture and highly viscous liquid.

Air-lift	High OTR Low shear stress Uniform mixing Easy scale-up	Dead zones of cells at high cell densities Serious foaming under high aeration. Poor oxygen mass transfer Poor mixing of fluid in a high cell density
RDR	High OTR Low shear stress Uniform mixing	Non-uniform mixing at a very large scale Difficult scale-up
Wave bioreactor	Disposable High mass and OTR Low-shear environment Bubble-free aeration Cost and time savings, Low level of foaming, Low risk of contamination Easy operation	Difficult scale-up Low heat transfer rate Difficult to apply advanced cultivation strategies
Membrane Bioreactor	High cell density High protein productivity Low stress to shear	Difficult scale-up Poor viability of cells Poor process stability Product heterogeneity

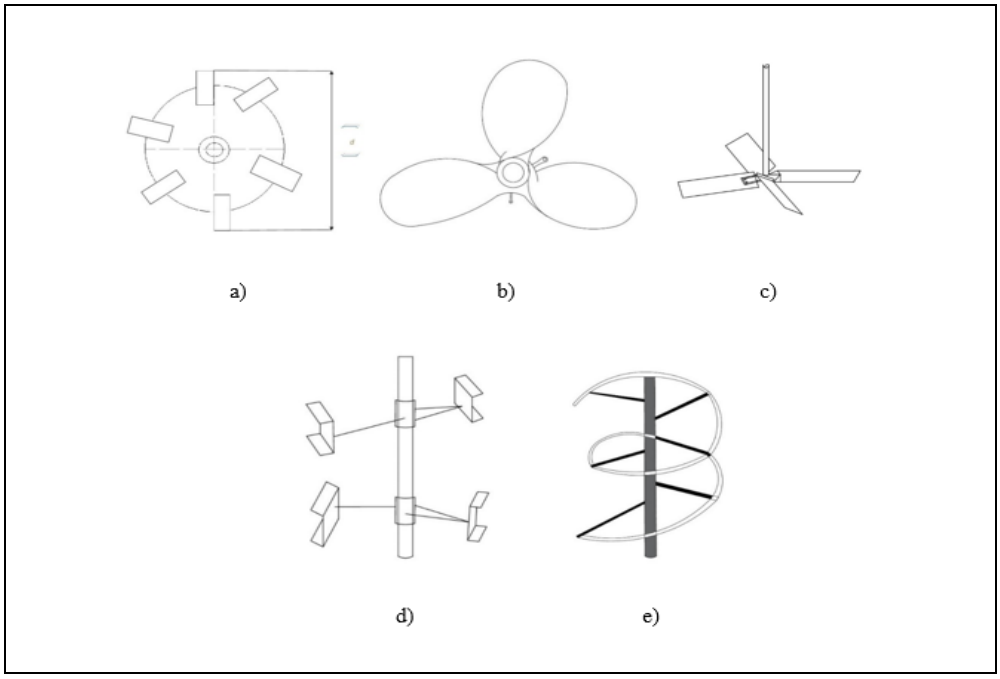


Figure 2.10. Impellers used for PCSC. (a) Rushton turbine, (b) Marine, (c) pitched-blade, (d) Intermig, (e) helical-ribbon (108)

2.8.2. Bioreactor operating mode used for cultivation of plant cell suspension culture

The operating modes practiced in mammalian and microbial systems including batch, two-stage culture, fed-batch, perfusion culture, and continuous and semicontinuous mode, may be used in PCSC systems (Figure 2.11). The most appropriate operation mode for the production of SMs depends on the growth rate, foaming, aggregation, type of product, and whether it is an intracellular or extracellular product. Also, it is dependent on the relationship between the growth of cells and product formation (non-related growth or growth-related product).

Batch mode is a closed system where organisms are inoculated in a fixed volume of liquid medium. This mode is suitable for small production volumes. Inside the bioreactor, the growth conditions vary constantly with nutrient depletion and at the same time with product accumulation. In fed-batch mode, nutrients are added progressively to the reactor and the products are harvested at the end of the production. Fed-batch mode is more flexible for selecting optimal conditions. Using fed-batch mode is possible to control both the reaction rate and metabolic reaction by the substrate feeding rate. Control of the reaction rate help to avoid problems caused by cooling and oxygen transfer. In continuous mode, fresh medium is continuously added and at the same time, an equal volume of cells is removed. The advantages of continuous mode are high productivity and better product quality due to steady conditions. On the other hand, the continuous mode requires flow control and stability of organisms (225) (235) (236) (237). Two-stage batch culture may be helpful for products that are not growth-associated (221). Two-stage culture can be managed in one bioreactor or in two separate bioreactors where the first bioreactor uses a growth medium for biomass production, while the second bioreactor is focused on product formation using a specific production medium where cell division is minimal or no cell division occurs (213) (225) (236) (238).

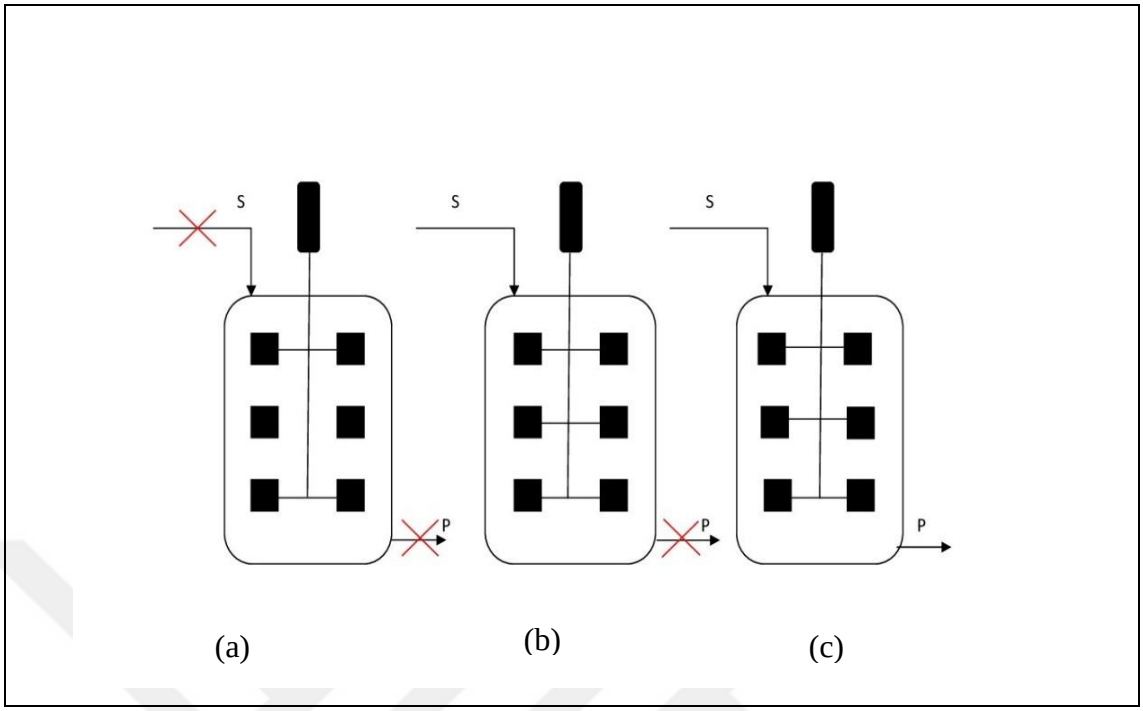


Figure 2.11. Operation modes for the cultivation of PCSC. (a) Batch, (b) Fed-Batch, and (c) Continuous

2.8.3. Secondary metabolites produced via bioreactors using plant cell suspension culture

For the successful production of the SMs from PCSC, many bioreactors have been designed and are successfully being used. A plant bioreactor's design is dependent on the properties of plant cell suspension. Choose of suitable bioreactor for PCSC cultivation into a bioreactor is dependent on oxygen transfer, fluid mixing, and the shear rate magnitude. Due to their suitable features for the cultivation of PCSC, STR, airlift bioreactors, bubble columns, and RDR were used for SMs production. In the literature, systems with capacities ranging from 1-2 L for lab use to 20.000 L for industrial use are found for producing PSMs using various bioreactors. (Table 2.9). The production of shikonin by Tabata and co-workers has a special place in the history of PCC cultivation. Ginseng is another well-known SM successfully produced at a very large scale (20 000 L) (Table 2.9). Table 2.9 lists bioreactors and their estimated volume used to cultivate PCSC. The largest culture, up to 75,000 L (*Echinacea purpurea)* has been cultivated in STR. The airlift bioreactors were used for the *Morinda citrifolia* and *C. roseus* plant cell growth in 1977 for the first time (239).

Table 2.9. Bioreactor operating mode used for cultivation of PCSC.

Bioreactor	**Operation Mode**	**Volume (L)**	**Plant Cell**	**Product**	**Product mg/L**	**Ref.**
STR	Batch	2.5	*Anchusa officinalis*	Rosmarinic Acid	3500	(240)
Jar culture vessel	Continuous	500	*Aralia cordata*	Anthocyanin	1090	(241)
Air-lift bioreactor	Batch	20	*Catharanthus roseus*	Ajmalicine Catharantine Serpentine Tryptamine	6.4 3 1.6 16.1	(36)
STR	Batch	14	*Catharanthus roseus*	Ajmalicine		(242)
Airlift reactor		20	*Catharanthus roseus*	Ajmalicine	0.323	(243)
STR	Batch Fed-batch Continuous	2,5	*Coptis japonica*	Berberine	800 2320 3500	(244)
STR	Batch	6	*Holarrhena antidysenterica*	Conessine	106	(245)
STR	Two-stage culture	200 500 750	*Lithospermum erythrorhizon*	Shikonin	4000	(246)
STR	Batch Fed-Batch		*Nicotiana tabacum*	Cinnamoyl putrescines	160 400	(247)
STR		20,000	*Nicotiana tabacum*	Nicotine, biomass		(248)
Erlenmeyer flask	Batch	0.25	Panax notoginseng	Ginseng saponin	1570	(249)
Airlift bioreactor	Batch	1	*Panax notoginseng*	Ginseng saponin	3120	(250)
STR		2000 20, 000	*Panax ginseng*	Ginseng saponin	500 700	(251)
Erlenmeyer flask	Batch	0.5	*Perilla frutescens*	Anthocyanin	5800	(252)
STR	Batch Fed-batch (intermittent feeding)		*Podophyllum*	Podophyllotoxin	13.8 43.2 48.8	(77)

	Continuous cell retention		*hexandrum*			
Erlenmeyer flask		0.25	*Taxus chinensis*	Taxane	274.4	(253)
Erlenmeyer flask	Semi-continuous batch process (SCBP)	0.25	*Taxus chinensis.*	Paclitaxel	85.5	(254)
Erlenmeyer flask Wilson type bioreactor		0.25	*Taxus cuspidata*	Taxol	22	(255)
RDR		2.5-1000	*Lithospermum erythrorhizon*	Shikonin		(248)
Bubble Column		1.1/ 5.8	*Vitis Vinifera*	Stilbenes		(256) (257)
STR		5L	*Stizolobium hassjoo*	L-DOPA	20590	(87)

2.9. OBJECTIVE OF THE STUDY

Scale-up of PCSC is a suitable system that can be modified to enhance product yield and allow studying the kinetics of cell biomass and SMs production. PCSCs are favorable for scaling up production due to their faster growth cycles, the opportunity to control the supply of nutrients and the high degree of uniformity between individual cells.

Hence, the purpose of the study is to (1) conceptually design a process applicable to general SMs production using PCSC, and (2) optimize the parameters such as the media compositions, the aeration rates, the speed of mixing, and the use of elicitors.

As a reference (model) plant and secondary metabolite *V. vinifera* cv. Öküzgözü & *V. labrusca* cv. Isabel and t-resveratrol have been used for the optimization of the system, respectively. Knowing the importance and use of t-resveratrol in nutraceuticals, cosmetics, and pharmacy creates a need for its production, as well. The bioproduction of t-resveratrol by plant cell suspensions responding to elicitors such as ß-CD and MeJA, without genetic modification, represents a suitable strategy for its production in the bioreactor.

Culture parameters such as nutritional requirement, inoculum density, mixing, temperature, pH, and dissolved oxygen, have been studied and optimized in STR for biomass growth and t-resveratrol production. The bioreactor contained nutrient-rich media suitable for the growth of cells, and then for the induction of resveratrol production elicitors has been added (MeJA). Intra- and extracellular t-resveratrol were determined using HPLC, as well as the characteristics and growth parameters of the culture of *Vitis* cells.

3. MATERIALS

3.1. EQUIPMENT AND DISPOSABLES

ESCO Level II Biosafety Cabinet, Teflon-plated callus spoon, Steri 250 Glass Bead Sterilizer, Buchner Filter Flask, Stopper, and Filter Funnels, IsoLab Gas pump #1500081 and silicone pipe, Erlenmeyer flask (100 mL and 250 mL, 500 ml), Automatic pipette and tips, Serological pipette and tips, Pasteur pipette, Glass bottles with screw caps, Petri dishes, Aluminum folio, and stretch film, Wish MeXterile 60 Autoclave, Wisd Thermostable IR-150 Incubator and GROTECH G94 and G74 Plant Growth Chambers, DaihanScientific Thermostable IS-10RL Shaker, Thermo Scientific Genesys 10S UV/Vis Spectrophotometer and cuvettes, Eppendorf Centrifuge 5810 R, Beaker, Falcon tubes (15 mL and 50 mL), Eppendorf tubes (2 mL), Scalpel blade, Forceps and tweezers, Oven, Mettler-Toledo Sevencompact pH meter (Mettler Toledo, Sevencompact), Milli-Q Water Purification System, Shimadzu Balance, and weighing dishes, Wash bottle, Fume hood, HPLC column (Zorbax Eclipse xdb C18).

3.2. CHEMICALS

Duchefa Biochemie #M0245 Gamborg B5 media (including modified vitamins), Duchefa Biochemie #M0222 Murashige-Skoog media (including vitamins), Sigma Aldrich plant growth regulators (indole butyric acid, naphthaleneacetic acid, kinetin, gibberellic acid, 6-benzyl amino purine), Duchefa Biochemie Sucrose (crystallized), Duchefa Biochemie Casein hydrolysate (powder), Sigma Aldrich Plant Tissue Culture Agar, Sigma Aldrich Methanol (98% or 27N), Duzey Lab Ethanol (96% (v/v) and 70% (v/v)), Sigma Aldrich Hydrochloric acid (99% (v/v) and 1 M), Sigma Aldrich Sodium hydroxide (3 M), Sodium hypochlorite (<5% (v/v)), Sigma Aldrich Acetone, Elicitors (Sigma Aldrich MeJA #392707, Sigma Aldrich ß-CD # C4805), Sigma Aldrich Fluorescein diacetate # F7378, Sigma Aldrich Ethyl acetate #270989.

3.3. PLANT MATERIALS

Vitis labrusca (L.) cv. Isabel (grape berry skin) cell lines and *Vitis vinifera* (L.) cv. Öküzgözü (petiole and leaf) cell lines were constructed at Yeditepe University Plant Tissue Culture and Genetics/Plant Biotechnology Laboratories from the certified mother plants which were supplied commercially.

4. METHODS

4.1. STERILIZATION OF *V. LABRUSCA* CV. ISABEL FRUITS

The immature fruits of *V. labrusca* cv. Isabel was washed under running water for 15 minutes, then surface-sterilized with 70% (v/v) EtOH for 7 minutes and rinsed twice with sterile distilled water (dH_2O) for 1-minute intervals. The fruits were then placed into 2 % (v/v) NaOCl for 15 minutes and rinsed with sterile dH_2O for 1-minute intervals 5-7 times (Figure 4.1).

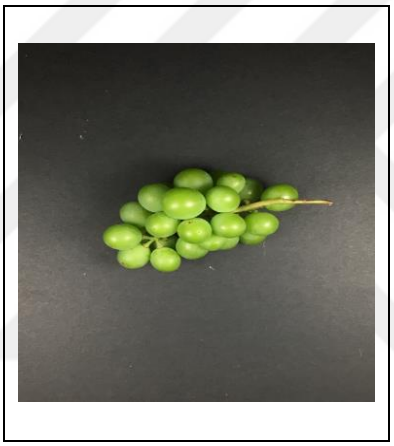


Figure 4.1. Surface sterilization of the *V. labrusca* cv. Isabel fruits.

4.2. CALLUS INDUCTION OF *V. LABRUSCA* CV. ISABEL

Callus induction was done by taking explants from the skin, exocarp, mesocarp, and endocarp. Explants were cut in 5 mm diameters and placed into VICIM4 medium supplemented with 3.164 g/L Gamborg B5 medium including vitamins, 20 g/L sucrose, 215 µg/L kinetin, 93µg/L NAA, and 6,8 g/L plant agar (Table 4.1). The pH was set to 5.7 before sterilization. Petri dishes containing *V.labrusca* cv. Isabel explants were maintained at 25°C under dark conditions. Some of the calli formations from skin explants were placed in the incubator at 25°C under 24 h light conditions to evaluate the response of explants to both dark and light conditions.

Table 4.1. Media composition for callus induction of *V. labrusca* cv. Isabel.

	B5 (g/L)	**Sucrose (g/L)**	**CH (mg/L)**	**NAA (µg/L)**	**Kinetin (µg/L)**	**Plant agar (g/L)**
VlCIM1	3.164	30	250	93	215	6.8
VlCIM1'	3.164	20	250	93	215	6.8
VlCIM4	3.164	20	-	93	215	6.8

4.3. ESTABLISHMENT OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

V. labrusca cv. Isabel CSCs were initiated by inoculating 2 g FW of callus pieces in 250 ml Erlenmeyer containing 50 ml of VlSCIM6 medium adjusted to pH=5.7. The flasks were maintained in a rotary shaker at 110 rpm at 25 °C under dark (2 Flasks) and light conditions (2 Flasks). Later, *V.labrusca* cv. Isabel CSCs were initiated also in some other media compositions to see the effect on cell growth (Table 4.2). The effect of CH on cell growth of *V. labrusca* cv. Isabel was studied using VlSCIM2, VlSCIM3, and VlSCIM6 media compositions.

Table 4.2. Media composition for *V.labrusca* cv. Isabel CSC (136) (210) (258).

	B5 (g/L)	**MS (g/L)**	**Sucrose (g/L)**	**CH (mg/L)**	**NAA (µg/L)**	**2.4-D (µg/L)**	**BAP (µg/L)**	**Kinetin (µg/L)**
VlSCIM2	3.164	-	30	250	100	-	-	200
VlSCIM3	3.164	-	30	-	100	-	-	200
VlSCIM6	3.164	-	20	-	100	-	-	200
VlSCIM7	3.164	-	20	-	-	2000	-	750
VlSCIM8'	-	4.405	30	-	-	100	1000	-
VlSCIM12	-	4.405	30	-	-	200	500	-

4.4. DETERMINATION OF CELL GROWTH (FW AND DW) AND PH VALUE FOR *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

First study

V. labrusca cv. Isabel CSC growth curve construction was performed using 7-day-old cells in triplicate at the second week of subculture maintained into VlSCIM2 media composition (Table 4.3). At this stage, 1 g of FW was washed with dH_2O and transferred into 100 ml flasks, and suspended in 20 ml of VlSCIM2 media. The growth of cell culture for *Vitis labrusca* cv. Isabel was determined by measuring FW, DW, and pH values. The cell culture was filtrated under a vacuum. The media were collected and kept at -20 °C for sugar measurement and the cells were weighed as FW. The fresh cells were dried at 85 °C for 24 h in the oven, and then DW was determined (174). The growth of *V.labrusca* cv. Isabel CSC was monitored for 13 days of cultivation and maintained in darkness. Every 2 days until day 13, the cells were harvested.

After the collection of media from the vacuum filtration, pH was measured for each sample by using a pH meter. Since an increase in cell water content results in an increase in fresh weight or cell size, the ratio of FW/DW was used as a measure of cell size or cell water content (259).

Second study

Growth curve construction for *V. labrusca* cv. Isabel CSC was performed in duplicate using 7-day-old cells maintained in VlSCIM2 media composition (Table 2.1). At this stage, 1 g of FW was washed with dH_2O and transferred into 100 ml flasks, and suspended in 20 ml of fresh growth VlSCIM2 media containing. The growth of cell culture for *V. labrusca* cv Isabel was determined by measuring FW, DW, and pH values. The cell culture was filtrated under a vacuum. The media were collected and kept at -20 °C for sugar measurement and the cells were weighed as FW. The fresh cells were dried at 85 °C for 24 h in an oven, and then DW was determined (174). The growth of *V.labrusca* cv. Isabel CSC was monitored for 31 days of cultivation and maintained in darkness. Every 2 days until day 31, the cells were harvested. pH was measured for each sample by using a pH meter.

Third study

The effect of CH on the cell growth of *V.labrusca* cv. Isabel was studied by using VlSCIM3 (Table 4.3). The cells from the VlSCIM2 media with casein were transformed into VlSCIM3. The growth of cell culture for *V. labrusca* cv. Isabel was determined by measuring FW, DW, and pH values. The cell culture was filtrated under a vacuum. The media were collected and kept at -20 ℃ for sugar measurement and the cells were weighed as FW. The fresh cells were dried at 85 ℃ for 24 h in an oven, and then DW was determined (174). The growth of *V. labrusca* cv. Isabel CSC was monitored for 17 days of cultivation and maintained in darkness. pH was measured for each sample by using a pH meter.

4.5. CELL DENSITY ANALYSIS OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

This study aimed to analyze the influence of initial cell density and correlation with media volume (g FW/media volume) on the growth dynamics of *V.labrusca* cv. Isabel CSC. Different concentrations of initial cells (2, 2,5, and 3 g FW cells) were transferred into 250 ml Erlenmeyer and suspended in 50 ml B5 media based on the article (176). The subculturing was performed at 7-day and cells were measured as fresh weight. The media volume was increased from 50 ml up to 100 ml media using a 250 ml Erlenmeyer, and 4,5 g (initial cell density) of cells was added. The subculturing was performed at 7-day and cells were measured as FW. Further, the volume of Erlenmeyer was changed from 250 ml to 500 ml and media volume was increased from 100 ml to 200 ml B5 media. The initial cell density was 9 g FW cells/200 ml B5 media (calculation: 4,5g FW per 100 ml; X g FW per 200 ml) X= 9 g FW/200 ml media.

4.5.1. Statistical Analysis

The correlation between initial density and media volume in *V. labrusca* cv. Isabel's CSC was analyzed. The results were analyzed statistically to determine the significant differences using Student's t-test. The null hypothesis, H0, is postulated as "initial density *does not correlate* with media volume". The alternative hypothesis, H1, is postulated as "Ho: initial density *correlates* with media volume". Type 3 (different groups with different variance) and two-tailed Student's t-test were performed with a significance level α of 0,05 and 0,01

($p < 0,05$ and $p < 0,01$). Changed variables are the initial density concentration and the effect of media volume.

4.6. EFFECT OF SUCROSE CONCENTRATION ON CELL GROWTH OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

The different concentration of sucrose was added to the VlSCIM2 media composition to analyze the growth curve of cells (Table 4.3). Pre-cultured 7-day-old CSC of *V. labrusca* cv. Isabel was filtered under vacuum and 1 g cells were transferred in a 100 ml Erlenmeyer with 20 ml VlSCIM2' and VlSCIM2'' media in triplicate for each measurement. The Erlenmeyers were incubated on a rotary shaker at 110 rpm under dark conditions in, a temperature-controlled room at 25±1°C.

Table 4.3. Sucrose concentration into VlSCIM2 media composition.

	B5 (g/L)	Sucrose (g/L)	CH (mg/L)	NAA (µg/L)	Kinetin (µg/L)
VlSCIM2	3.164	30	250	100	200
VlSCIM2'	3.164	40	250	100	200
VlSCIM2''	3.164	50	250	100	200

4.7. ELICITOR TREATMENT PROCEDURE ON *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

First study

Pre-cultured 7-day-old cell suspensions of *V. labrusca* cv. Isabel was filtered under vacuum and 1 g cells were transferred in a 100 ml Erlenmeyer with 20 ml VlSCIM2 media in triplicate for each measurement. The Erlenmeyers were incubated on a rotary shaker at 110 rpm under dark conditions in a temperature-controlled room at 25±1°C. To stimulate resveratrol production, CSC of *V. labrusca* cv. Isabel was elicited with MejA and with a combination of MeJA and ß-CDs (Table 4.4). The MeJA was first dissolved in 96% EtOH and after the filter sterilization (45 µm syringe filter) was added into autoclaved media at

100 µM concentration. MeJA was added to the CSC at the beginning of their log phase of growth (124) except when the combination of them is used. ß -CDs were dissolved in 1M NH_4OH and after the filter sterilization (45 µm syringe filter) were added into autoclaved media at 50 mM concentration. ß-CDs were added into media on the first day in all Erlenemyers, whereas MeJA was added on 6th day of subculturing. The combined concentration between them was 100 µM MeJA +50 mM ß-CDs. The cells were harvested every 2 days until day 13.

Table 4.4. Elicitor treatment in *V.labrusca* cv. Isabel CSC (First study).

Days of Fermentation	Elicitors TIME (sampling)	Control	MeJA (100µM)	MeJA ((100µM)+ ß-CDs (50 Mm)
1	Day 1	C1, C2, C3	M1, M2, M3	ß1, ß2, ß3 ß-CD was added
3	Day 3	C1, C2, C3	M1, M2, M3	ß1, ß2, ß3
5	Day 5	C1,C2, C3	M1,M2, M3	Mß1, Mß2, Mß3
6	Day 6		MeJA was added	MeJA was added
7	Day 7	C1,C2, C3	M1,M2, M3	Mß1, Mß2, Mß3
9	Day 9	C1, C2, C3	M1, M2,M3	Mß1, Mß2, Mß3
11	Day 11	C1, C2, C3	M1,M2, M3	Mß1, Mß2, Mß3
13	Day 13	C1, C2, C3	M1,M2, M3	Mß1, Mß2, Mß3

Second study

To stimulate t-resveratrol production, CSC of *V. labrusca* cv. Isabel was elicited with MeJA, ß-CDs, and a combination of MeJA and ß-CDs. The MeJA was first dissolved in 96% EtOH and after the filter sterilization (45 µm syringe filter) was added into autoclaved media at 100 µM concentration. ß -CDs were dissolved in 1M NH_4OH and after the filter sterilization (45 µm syringe filter) were added into autoclaved media at 50 mM concentration. MeJA and ß-CDs were added to the CSC at the beginning of their log phase of growth (on the 5th day) (124). When the ß-CDs were combined with MeJA, they were added to the media on the 6th day of their growth (Table 4.5). The combined concentration between them was 100 µM MeJA +50 mM ß-CDs. The growth of *V. labrusca* cv. Isabel CSC using elicitors was monitored for 13 days of cultivation and maintained in darkness. The cells were harvested every 2 days until day 13. The media were collected and kept at -20 °C for extracellular t-

resveratrol and sugar analysis. Cells were measured and used for intracellular resveratrol analysis.

Table 4.5. Elicitor treatment in *V. labrusca* cv. Isabel CSC (Second study).

Days of Fermentation	Elicitors TIME (sampling)	Control	MeJA (100µM)	ß-CDs (50 Mm)	MeJA ((100µM)+ ß-CDs (50 Mm)
1	Day 1	C1, C2,	C1, C2,	C1, C2,	C1, C2,
3	Day 3	C1, C2,	C1, C2,	C1, C2,	C1, C2,
5	Day 5	C1, C2,	MeJA was added	ß-CDs were added	C1, C2,
6	Day 6				MeJA + ß-CDs were added
7	Day 7	C1, C2,	M1, M2,	ß1, ß2,	Mß1, Mß2,
9	Day 9	C1, C2,	M1, M2,	ß1, ß2,	Mß1, Mß2,
11	Day 11	C1, C2,	M1, M2,	ß1, ß2,	Mß1, Mß2,
13	Day 13	C1, C2,	M1, M2,	ß1, ß2,	Mß1, Mß2,

4.8. CELL VIABILITY TEST OF *V. LABRUSCA* CV. ISABEL CELLS WITH FLUORESCENCE MICROSCOPY

The Fluorescein diacetate test (FDA) was used for cell viability measurement. Twenty-five milligrams of FDA were dissolved with 5 ml acetone. In a laminar flow, 950 µL of the sample (7 – day-old cells) was transferred from continuous *V. labrusca* cv. Isabel CSC to 2 ml Eppendorf. 1 ml of VlSCIM2 media was added in 2 ml Eppendorf tubes that contain cell suspensions. Then, 50 µL of FDA was added into 2 ml Eppendorf. Eppendorf tubes were covered with aluminum foil and incubated for 5 minutes at room temperature. FDA is digested by esterases within living cells, releasing the fluorescein molecule, which lights up under UV light. After 5 minutes, a drop of the sample was taken and put into the microscope slide. A cover slide was used to cover the slide. Living cells were detected by using a fluorescence microscope (202).

4.9. EXTRACELLULAR TRANS-RESVERATROL EXTRACTION FROM *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

Five milliliters of the sample were mixed with 5 mL of 100% ethyl acetate and for 5 minutes were mixed to extract extracellular t-resveratrol. The mixture was allowed to settle for 15 minutes at room temperature then the upper phase was collected. The removed lower phase was re-extracted by adding 5 ml ethyl acetate two times. The ethyl acetate was removed from the organic phase by rotary evaporation at 35 °C for around 3h. The pellet was resuspended in 1ml MeOH and kept at -20°C for HPLC analysis. All procedure was performed in dark (124) (174) (260) (Figure 4.2).

4.10. INTRACELLULAR TRANS-RESVERATROL EXTRACTION FROM *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

Four milliliters of methanol were used to extract 1 g of fresh cells. The mixtures were extracted all night at room temperature in a rotary shaker at 110 rpm, to define the amount of intracellular t-resveratrol. After that, the extract was clarified by centrifugation at 14,000xg for 10 min. The samples were kept at -20°C for HPLC analysis. All procedure was performed in dark (176) (Figure 4.2).

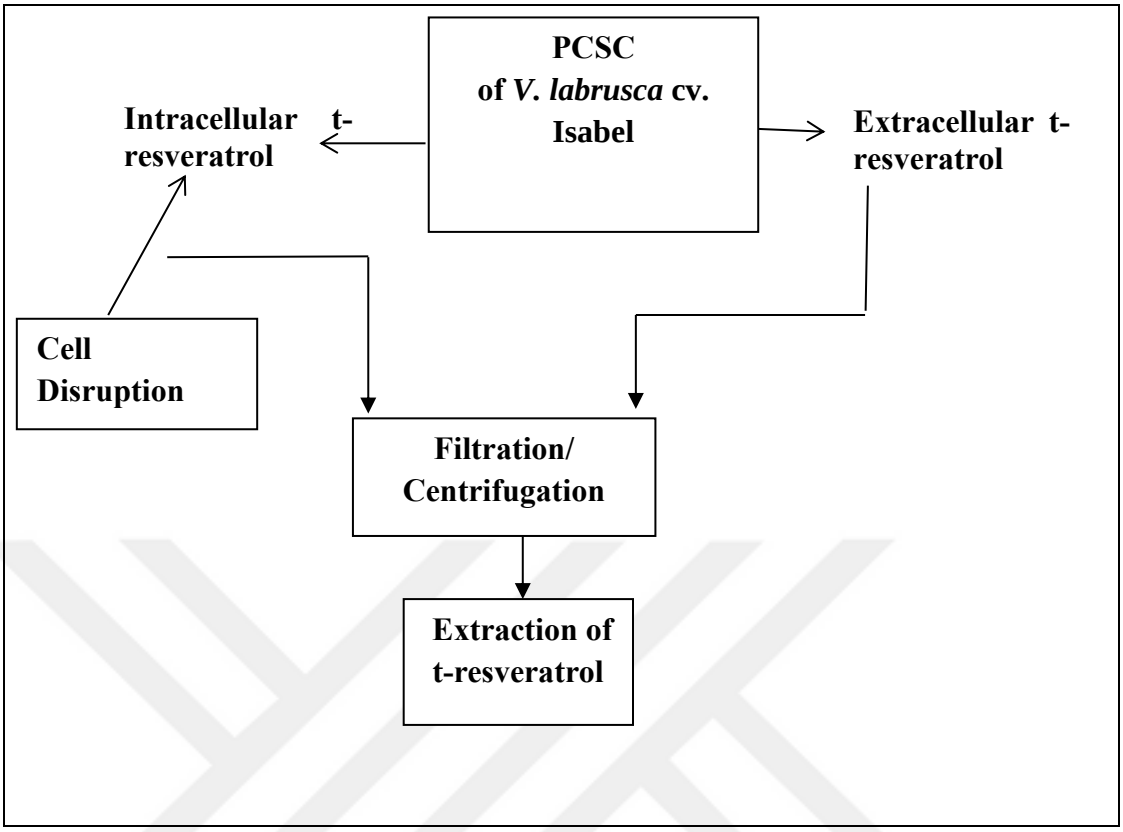


Figure 4.2. Downstream Processes for intra and extracellular t-resveratrol production (261)

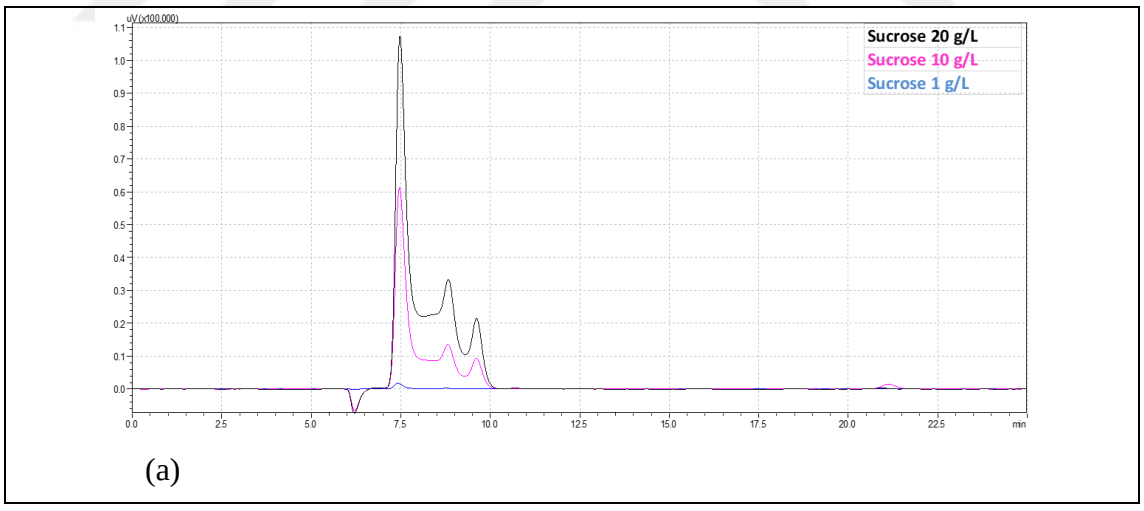
4.10.1. HPLC analysis of extra/intracellular t-resveratrol production from *V. labrusca* cv. Isabel cell suspension culture

The t-resveratrol analysis was performed on HPLC (Column: Agilent ZORBAX Eclipse XDB columns C18) according to the procedure from Zamboni et.al, (2006) (176). Owing to the column diameters used in this article being different from the own column we used, the injection rate and flow rate were changed. The flow rate was 0.7 ml/min and the injection volume was 25 µl. The UV-VIS spectra were recorded at 310 nm. The mobile phases are composed of 0.1 % acetic acid in H_2O (A) and acetonitrile (B). All mobile phase was filtered through 0.22 µm RC (Regenerated cellulose) membrane to remove particulate matter. The following conditions were used for the 30-minute separation process at 40 °C: linear gradients starting at 5% B, to 70 % B in 25 min, to 95 % B in 0.1 min, 95 % B for 2 min, and back to 5 % B in 0.1 min. The concentrations of resveratrol were prepared to a range from 0.1-10 mg/L by dissolving in MeOH (HPLC grade). The standards and samples were kept at -20 °C until HPLC analysis.

4.11. SUGAR ANALYSIS FOR *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

FIRST STUDY

The residual sugar contents in *V. labrusca* cv. Isabel suspension medium was measured by HPLC (LC Shimadzu) using Aminex HPX-87H HPLC, 300 x 7.8 mm column by refractive index detector, at a working flow rate of 0.6 ml/min at 50°C using 5 mM H_2SO_4 as the mobile phase. The samples were filtered through a 0.22 µm RC membrane filter. Each sample was injected twice with a 20 µL injection volume and the peak was detected. Different concentrations (20, 10, 1.0, 0.5, 0.25, and 0.1 g/L) of sucrose, glucose, and fructose were used for standard curve construction. The remaining concentrations of sucrose, fructose, and glucose in the samples were identified by comparing their counted peak area with the standard curves. The retention times for sucrose, fructose, and glucose were 7.412, 9.598, and 8.801 minutes, respectively (Figure 4.3).



(a)

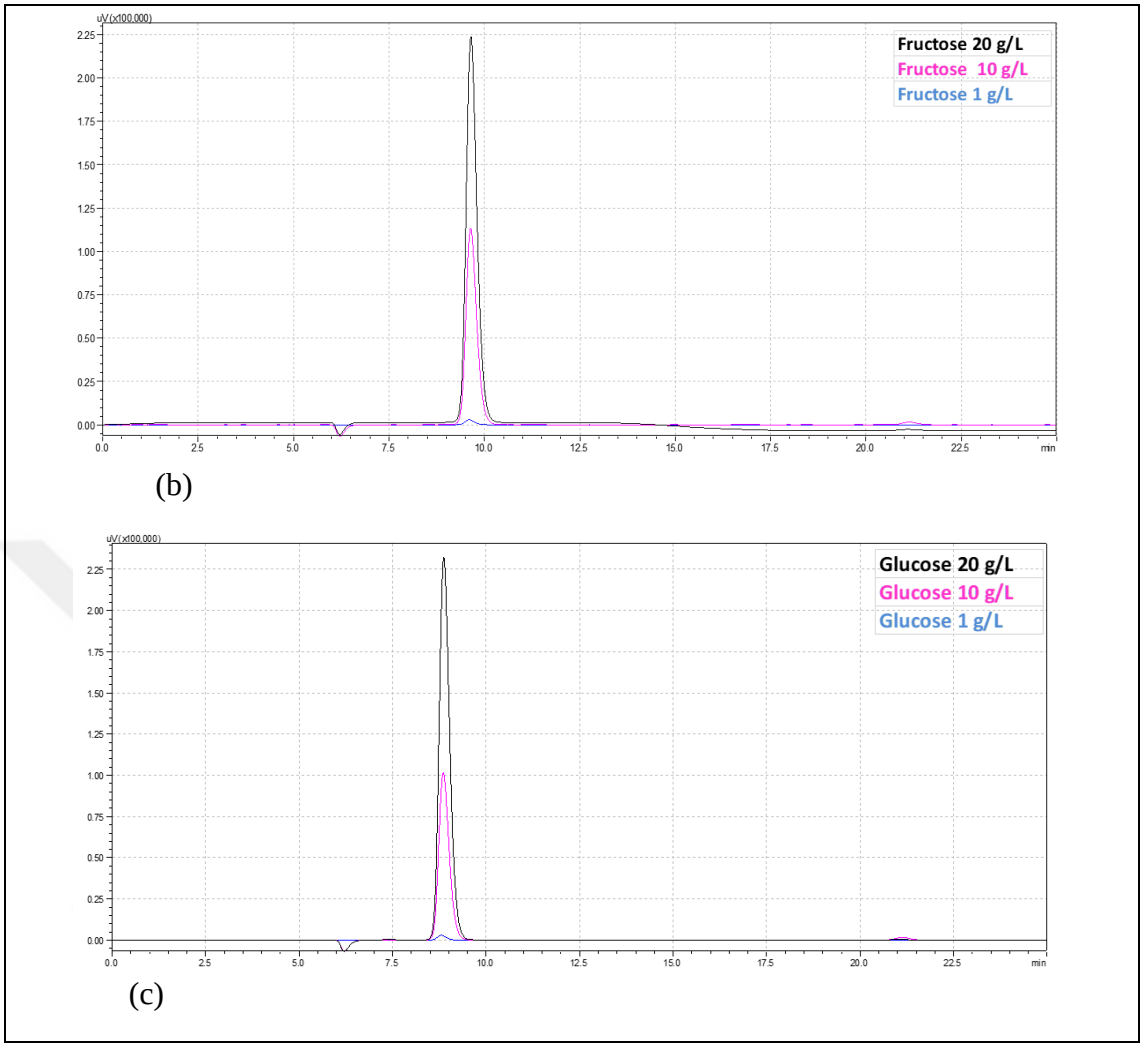


Figure 4.3. Chromatograms of sugar standards (20, 10, and 1 g/L). (a) Sucrose, (b) Fructose, and (c) Fructose for *V. labrusca* cv. Isabel (First study)

SECOND STUDY

The residual sugar contents in *V. labrusca* cv. suspension medium was measured by using HPLC (LC Shimadzu) using Aminex HPX-87H HPLC, 300 x 7.8 mm column by refractive index detector, at a working flow rate of 0.6 ml/min at 50°C using 5 mM H_2SO_4 as the mobile phase. The samples were filtered through a 0.22 µm RC membrane filter. Each sample was injected twice with a 20 µL injection volume and the peak was detected. Different concentrations (40, 30, 20, and 10 g/L) of sucrose, glucose, and fructose were used for standard curve construction. The remaining concentrations of sucrose, fructose, and glucose in the samples were identified by comparing their counted peak area with the standard

curves. The retention times for sucrose, fructose, and glucose were 7.481, 9.652, and 8.873 minutes, respectively (Figure 4.4).

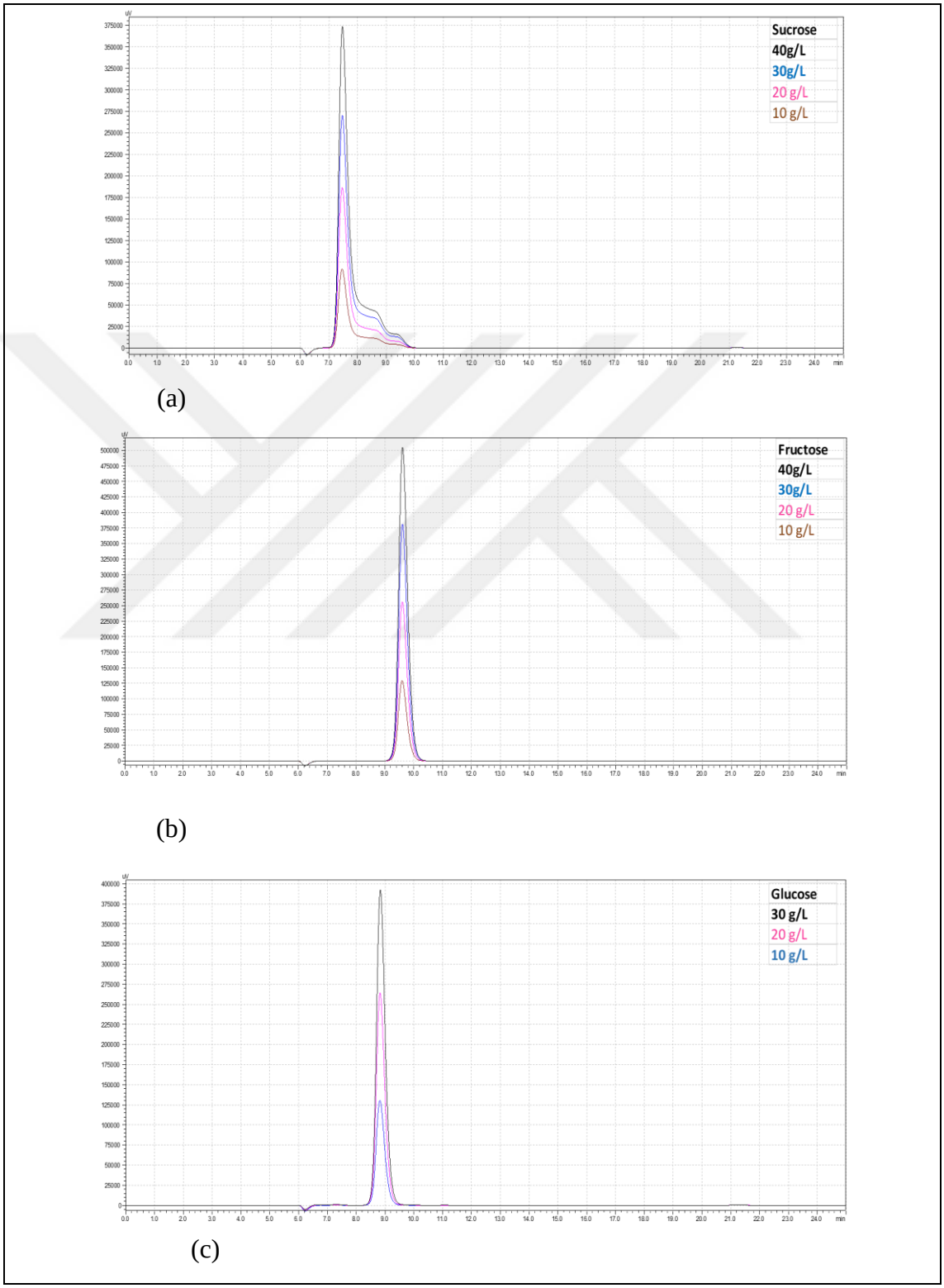


Figure 4.4. Chromatograms of sugars standards 40, 30, 20, and 10 g/L. (a) Sucrose, (b) Fructose, and (c) Glucose for *V. labrusca* cv. Isabel (Second study)

4.12. PROCESS SCALE-UP OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE IN STR

To stimulate biomass and t-resveratrol production in the bioreactor, CSC of *V. labrusca* cv. Isabel was used. Scale-up of grapevine cell suspensions for t-resveratrol production in bioreactor requires optimization of many parameters such as (135):

1. The aeration rates range from 0.025-2 vvm
2. The speed of mixing ranges from 50 -100 rpm
3. The density of inoculum range from 10-50 g/L
4. The concentration of sugar range from 20-60 g/L.

Considering the culture medium (micro- & macro-element and plant growth hormone concentrations) set in Erlenmeyer, the others parameter (1, 2, 3, 4) were considered for the scale-up of *V. labrusca* cv. Isabel cells in the bioreactor.

4.12.1. Bioreactor conditions

First study

For this study, a CSC of *V.labrusca* cv. Isabel was used. STR culture experiments were conducted for 5 days (except trial 2 and trial 7 which were conducted for 3 and 6 days, respectively). The bioreactor culture was initiated by inoculating a 7-day-old CSC into a 6 L bioreactor (455 mm x 375 mm x 740 mm (W x D x H) containing 3 L of VlSCIM2 media (3.164 g/L Gamborg B5 including vitamins, 30 g/L sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L NAA, and 0.2 mg/L kinetin). The mixing (two Rushton turbine impellers) was set to 75 rpm (except in trial 3, it was set to 50 rpm) based on articles (1) (193). The aeration rate was 0.02 vvm (0.06 L/min) initially in all trials, then it was increased in the right balance with the oxygen consumption by cells. DO was kept above the critical oxygen concentration (15-20%) at 30 % to avoid oxygen limitation (262). pH probe was calibrated before sterilization and the oxygen probe was calibrated to 100 percent with the use of the sparger. pH was set at 6.0 (except in trial 1, which was set at 5.7). The cells were then transferred using a glass funnel with heat sterilization. The cells were cultured in darkness at 25 °C

(except in trials 5 and 7 which were conducted in light). Table 4.6 details how the culture parameters for each trial were changed to enhance cell development.

Table 4.6. Optimization of parameters in *V.labrusca* cv. Isabel CSC scale-up studies (First study).

Nr of trials	Temperature (°C)	Mixing (rpm)	pH	pO_2 (%)	Airflow (L/min)	Dark/Light	Inoculant size (g/3L B5 media)	Batch time (days)
1	25	75	5.7	>30	0.06	Dark	60	5
2	25	75	6.0	>30	0.45	Dark	60	3
3	25	50	6.0	>30	0.24	Dark	60	5
4	25	75	6.0	>30	0.06-0.24	Dark	135	5
5	25	75	6.0	>30	0.06-0.24	Light	135	5
7	25	75	6.0	>30	0.06-0.45	Light	135	6
8	25	75	6.0	>30	0.06-0.45	Dark	135	5
9	25	75	6.0	>30	0.06-0.45	Light	135	6
10	25	75	6.0	>30	0.06-0.45	Dark	135	5
11	25	75	6.0	>30	0.06-0.8	Dark	135	5
12	25	75	6.0	>30	0.06-0.8	Dark	135	5
13	25	75	6.0	>30	0.06-0.8	Dark	135	5
14	25	75	6.0	>30	0.06-0.8	Dark	135	5
17	25	75	6.0	>30	0.06-0.8	Dark	135	5

Second study

The effect of CH on the cell growth of *V. labrusca* cv. Isabel in an STR was studied by using VlSCIM3. Bioreactor culture experiments were conducted for 8 days. The bioreactor culture was initiated by inoculating a 7-day-old cell suspension into a 6 L bioreactor (455 mm x 375 mm x 740 mm (W x D x H) containing 3 L of VlSCIM3 media (3.164 g/L Gamborg B5

including vitamins, 30 g/L Sucrose, 0.1 mg/L NAA, and 0.2 mg/L kinetin). The mixing (two Rushton turbine impellers) was set to 75 rpm based on articles (1) (193). The aeration rate was 0.02 vvm (0.06 L/min) initially in two trials, then it was increased in the right balance with the oxygen consumption by cells. DO was kept above the critical oxygen concentration (15-20%) at 30 % to avoid oxygen limitation (262). pH probe was calibrated before sterilization and the oxygen probe was calibrated to 100 percent with the use of the sparger. pH was set at 6.0. The cells were then transferred using a glass funnel with heat sterilization. The cells were cultured in darkness at 25 °C. Table 4.7 details how the culture parameters for each trial were changed to enhance cell development.

Table 4.7. Optimization of parameters in *V. labrusca* cv. Isabel scale-up studies (Second study).

Nr of trials	**Temperature (°C)**	**Mixing (rpm)**	**pH**	**pO_2 (%)**	**Airflow (L/min)**	**Dark/Light**	**Inoculant size (g/3L B5 media)**	**Batch time (days)**
Trial 15	25	75	6.0	>30	0.06-0.8	Dark	135	8
Trial 16	25	75	6.0	>30	0.06-0.8	Dark	135	8

4.13. ELICITOR TREATMENT PROCEDURE ON *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE IN STR

For this study, the CSC of *V. labrusca* cv. Isabel was conducted for 5 days in the bioreactor. The bioreactor culture was initiated by inoculating a 7-day-old cell suspension into a 6 L bioreactor (455 mm x 375 mm x 740 mm (W x D x H) containing 3 L of VlSCIM2 media (3.164 g/L Gamborg B5 including vitamins, 30 g/L Sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L NAA, and 0.2 mg/L kinetin) (table 4.8). The mixing (two Rushton turbine impellers) was set to 75 rpm based on articles (1) (193). The aeration rate was 0.02 vvm (0.06 L/min) initially then it was increased in the right balance with the oxygen consumption by cells. Dissolved oxygen was kept above the critical oxygen concentration (15-20%) at 30

% to avoid oxygen limitation (262). pH probe was calibrated before sterilization and the oxygen probe was calibrated to 100 percent with the use of the sparger. pH was set at 6.0. The cells were then transferred using a glass funnel with heat sterilization. The cells were cultured in darkness at 25 °C (Table 4.8). After 48 hours, the cells were elicited with the addition of MeJA (100µM). The MeJA was first dissolved in 96% ethanol and after the filter sterilization (45 µm syringe filter) was added into autoclaved media.

Table 4.8. Optimization of parameters in *V. labrusca* cv. Isabel CSC scale-up studies with elicitor.

Nr of trials	Temperature (°C)	Mixing (rpm)	pH	pO_2 (%)	Airflow (L/min)	Dark/ Light	Inoculant size (g/3L B5 media)	Batch time (days)
Trial 18	25	75	6.0	>30	0.06-0.8	Dark	135	5

4.14. SUGAR ANALYSIS FOR *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE CULTIVATED IN STR

The residual sugar contents in *V.labrusca* cv. CSC medium was measured by HPLC (LC Shimadzu) using Aminex HPX-87H HPLC, 300 x 7.8 mm column by refractive index detector, at a working flow rate of 0.6 ml/min at 50°C using 5 mM H_2SO_4 as the mobile phase. The samples were filtered through a 0.22 µm RC membrane filter. Each sample was injected twice with a 20 µL injection volume and the peak was detected. Different concentrations (30, 15, 7.5, 5, and 1 g/L) of sucrose, glucose, and fructose were used for standard curve construction. The remaining concentrations of sucrose, glucose, and fructose in the samples were identified by comparing their counted peak area with the standard curves. The retention times for sucrose, glucose, and fructose were 7.481, 8.873, and 9.652 minutes, respectively.

4.15. CELL VIABILITY TEST OF *V. LABRUSCA* CV. ISABEL CELLS WITH FLUORESCENCE MICROSCOPY

The Fluorescein diacetate test (FDA) was used for cell viability measurement. Twenty-five milligrams of FDA were dissolved with 5 ml acetone. Using heat sterilization, 950 µL of the sample (7 – day-old cells) was transferred from continuous *V. labrusca* cv. Isabel CSC in the bioreactor to 2 ml Eppendorf. 1 ml of VlSCIM2/VlSCIM3 media was added in 2 ml Eppendorf tubes that contain cell suspensions. Then, 50 µL of FDA was added into 2 ml Eppendorf. Eppendorf tubes were covered with aluminum foil and incubated for 5 minutes at room temperature. FDA is digested by esterases within living cells, releasing the fluorescein molecule, which lights up under UV light. After 5 minutes, a drop of the sample was taken and put into the microscope slide. A cover slide was used to cover the slide. Living cells were detected by using a fluorescence microscope (202).

4.16. EXTRACELLULAR TRANS-RESVERATROL EXTRACTION FROM *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE IN STR

Five milliliters of the sample were mixed with 5 mL of 100% ethyl acetate and mixed for 5 min to extract extracellular t-resveratrol. The mixture was allowed to settle for 15 minutes at room temperature then the upper phase was collected. The removed lower phase was re-extracted by adding 5 ml ethyl acetate two times. The ethyl acetate was removed from the organic phase by rotary evaporation at 35 °C for around 3h. The pellet was resuspended in 1ml MeOH and kept at -20°C for HPLC analysis. All procedure was performed in the dark (124) (260) (174).

4.16.1. HPLC analysis of extra/intracellular trans-resveratrol production from *V. labrusca* cv. Isabel cell suspension culture in STR

The t-resveratrol analysis was performed on HPLC (Column: Agilent ZORBAX Eclipse XDB columns C18) according to the procedure from Zamboni et.al, (2006) (176). Owing to the column diameters used in this article being different from the own column we used, the injection rate and flow rate were changed. The flow rate was 0.7 ml/min and the injection

volume was 25 µl. The UV-VIS spectra were recorded at 310 nm. The mobile phases are composed of 0.1 % acetic acid in H_2O (A) and acetonitrile (B). All mobile phase was filtered through 0.22 µm RC (Regenerated cellulose) membrane to remove particulate matter. Separation was carried out at 40 °C in 30 min, under the following conditions: linear gradients starting at 5% B, to 70 % B in 25 min, to 95 % B in 0.1 min, 95 % B for 2 min, back to 5 % B in 0.1 min. The concentrations of resveratrol were prepared to a range from 0.1-10 mg/L by dissolving in MeOH (HPLC grade). The standards and samples were kept at -20 °C until HPLC analysis.

4.17. EXTRACTION OF ANTHOCYANIN FROM *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE CULTIVATED IN ERLENMEYER AND STR

The procedure was followed as described by Hirasuna *et al*. 1991. *V. labrusca* cv. Isabel media along with cells were taken from the bioreactor using a 15 ml Falcon tube. Samples in 15 mL falcon tubes were centrifuged in Eppendorf Centrifuge 5810 R at 1000G for 5 minutes. Supernatants were removed using serological pipettes, and cell pellets were used for anthocyanin analysis. *V. labrusca* cv. Isabel CSC from 250 ml Erlenmeyer was harvested by vacuum filtration on day 7 and maintained in both dark and light conditions and cell pellets were used for anthocyanin analysis. Five milliliters of acidic MeOH (1% concentrated (12 N) HCl by volume) was added to the cell pellet after the supernatant was removed, resuspended by vortex for 2 minutes, and allowed to stand all night at 4°C. After extraction, the samples were centrifuged in Eppendorf Centrifuge 5810 R at 1000G for 3 minutes to pellet cell debris. The supernatant fractions were analyzed spectrophotometrically at 525 nm with Thermo Scientific Genesys 10S UV/Vis Spectrophotometer. Acidic methanol (1:100 (v/v) HCl: CH_3OH) was used as blank. The results were recorded and averaged and anthocyanin concentration was calculated.

Calculate anthocyanin pigment concentration (red pigment), expressed as cyanidin-3-glucoside equivalents, as follows:

$$\text{Anthocyanin pigment(cyaniding-3-glucoside equivalents, mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

using the following formula that was formulated by Lee *et al.* 2005 where A is the absorbance, MW is the molecular weight of cyanidin-3-glucoside which is equal to 449,2 g/mol, DF is the dilution factor, ε is the molar extinction coefficient of cyanidin-3-glucoside which is equal to 26.900 $L\,mol^{-1}\,cm^{-1}$, 10^3 is the conversion factor from grams to milligrams, and lastly, 1 is the pathway length of the spectrophotometer (263) (264).

4.18. STERILIZATION OF *V.VINIFERA* CV. ÖKÜZGÖZÜ

4.18.1. Sterilization of *V. vinifera* cv. Öküzgözü leaves

The leaf tissues were surface-sterilized with 2% NaOCl for 10 minutes and then were rinsed 5 times with sterile dH_2O (Figure 4.5) (126).

4.18.2. Sterilization of *V. vinifera* cv. Öküzgözü petioles

The petioles were surfaced with 5% NaOCl for 10 minutes and then rinsed with sterile dH_2O 5 times (177) (202) (Figure 4.5).

Figure 4.5. Sterilization of *V. vinifera* cv. Öküzgözü

4.19. CALLUS INDUCTION OF *V. VINIFERA* CV. ÖKÜZGÖZÜ

Callus was induced from leaves and petioles of *V. vinifera* cv. Öküzgözü. After the sterilization process, explants were cut in 5 mm diameters and placed on plates with a solid growth B5 and MS media supplemented with different concentrations and combinations of growth regulators (NAA, kinetin, IAA, BAP, and 2,4-D). Also, the B5 and MS medium without growth regulators were used for callus induction as control (Table 4.9). During the present work, the concentration of growth regulators was optimized to get a better response to callus induction (Table 2.1). The pH was adjusted between 5.7 - 5.75 for each media before sterilization. Petri dishes containing *V. vinifera* cv. Öküzgözü explants were kept in the incubator at 25 °C in the dark conditions (Table 4.9) (Figure 4.6) (124) (169) (177).

Table 4.9. Media compositions for *V. vinifera* cv. Öküzgözü callus induction.

MEDIA COMPOSITIONS	B5 (g/L)	MS (g/L)	Sucrose (g/L)	CH (mg/L)	NAA (mg/L)	Kinetin (mg/L)	IAA (mg/L)	BAP (mg/L)	2,4-D (mg/L	Agar (g/L)	pH
Control Media I (VvCIM C1)	3.164		30	250						7	5.7
Control media II (VvCIM C2)	3.164		20	250						7	5.7
Control media III (VvCIMC3)	-	4.405	30	250						7	5.7
VvCIM1	3.164	-	30	250	0.1	0.2	-	-	-	7	5.7
VvCIM(1)	3.164	-	30	250	0.5		-	0.5	-	7	5.75
VvCIM(1')^{IAA}	3.164	-	30	250			0.5	0.5	-	7	5.75
VvCIM (2)	3.164	-	20	250	0.2	1	-	-	-	7	5.7
VvCIM(3)	3.164	-	20	250	1	0.2	-	-	-	7	5.7
VvCIM (4)	3.164	-	30	250	0.093	0.215	-	-	-	7	5.7
VvCIM (5)	-	4.405	30	250		-	-	0.5	1	7	5.7
VvCIM (6)	-	4.405	30	250		-	-	1	2	7	5.7
VvCIM(7)	-	4.405	30	-	0.2	-	-	0.3	2	7	5.75

4.20. FRESH WEIGHT ANALYSIS FOR *V. VINIFERA* CV. ÖKÜZGÖZÜ CALLUS

The growth of callus was monitored by measuring the FW of biomass grown in four different media (VvCIM1, VvCIM$^{IAA'}$, VvCIM2, VvCIM3, and VvCIM4), from both explant sources (petiole and leaf) consisted of five replicates for each of them (Figure 4.6). Data on callus FW were recorded every 10 days and callus were subcultured on fresh media every 20 days.

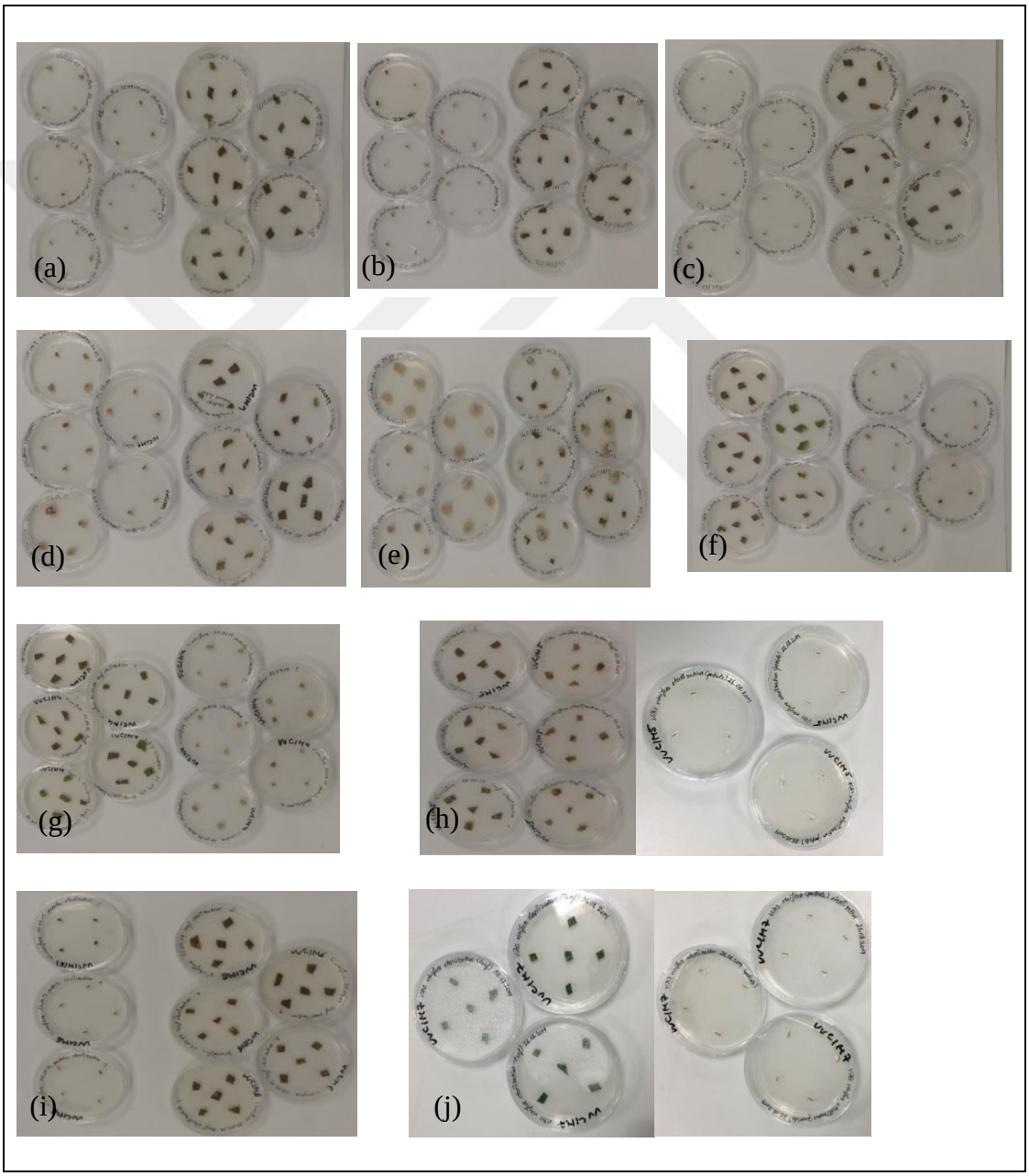


Figure 4.6. Callus induction from *V. vinifera* cv. Öküzgözü leaf and petiole explants. (a) VvCIM C1 media, (b) VvCIM C2 media, (c) VvCIM C3 media, (d) VvCIM1 media, (e)

VvCIM2 media, (f) VvCIM3 media, (g) VvCIM4 media, (h) VvCIM5 media, (i) VvCIM6 media, and (j) VvCIM7 media

4.20.1. Assessment of *V. vinifera* cv. Öküzgözü callus growth kinetics

Based on the fresh mass of *V. vinifera* cv. Öküzgözü for both explant sources of callus (petioles and leaves), callus relative fresh weight (CRFW), and callus relative growth rate (CRGR) were calculated according to the following formula:

CRFWG = ([W2 − W1]/W1)

CRGR = (lnW2 − lnW1)/Number of days (265)

Where W1 is the average of FW after 0 days of callus culture, and W2 is the average of FW after 10, 20, 30, and 40 days of callus culture.

4.21. ESTABLISHMENT OF *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

V.vinifera cv. Öküzgözü CSC was initiated by inoculating 2 g FW callus pieces in 250 ml Erlenmeyer containing 50 ml of B5 medium with different growth regulators adjusted to pH between 5.7-5.75, except one media (VVSCIM2) was adjusted to pH=6 (Table 4.10). The Erlenmeyers were kept in a rotary shaker at 110 rpm at 25 °C in dark conditions.

Table 4.10. Media composition for *V. vinifera* cv. Öküzgözü CSC.

Media Composition	B5 (g/L)	MS (g/L)	Sucrose (g/L)	CH (mg/L)	NAA(mg/L)	Kinetin (mg/L)	IAA (mg/L)	BAP (mg/L)	2,4-D (mg/L)	pH
VvSCIM1	3.164	-	30	250	0.1	0.2	-	-	-	5.7
VvSCIM2	3.164	-	20	250	0.1	0.2	-	-	-	6.0
VvSCIM3	3.164	-	30	250			0.5	0.5	-	5.75
VvSCIM4	3.164	-	20	250	1	0.2	-	-	-	5.7
VvSCIM5	3.164	-	30	250	-	-	-	0.5	0.2	5.75
VvSCIM6	3.164	-	30	250		-	-	1	0.2	5.75

VvSCIM7	3.164	-	20	250	0.2	1	-	-	-	5.75
VvSCIM8		4.405	30	250	-	-	-	1	0.1	5.75
VvSCIM8' (no CH)	-	4.405	30	-	-	-	-	1	0.1	5.75
VvSCIM9	3.164	-	30	250	1.5	-	-	1.5	-	5.75
VvSCIM10	3.164	-	30	250	0.5	-	-	0.5	-	5.75
VvSCIM11	3.164	-	30	250	0.5	-	-	2	-	5.75
VvSCIM12		4.405	30	-	-	-	-	0.5	0.2	5.75
VvSCIM13	3.164	-	20	-	1	0.2	-	-	-	5.7
VvSCIM14	3.164	-	30	-	0.1	0.2	-	-	-	5.75
VvSCIM15	-	4.405	30	-	6	-	-	0.6	-	5.7

4.22. DETERMINATION OF CELL GROWTH (FW AND DW) AND PH VALUE FOR *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

Growth curve construction for *V. vinifera* cv. Öküzgözü was performed in duplicate using 7-day-old cells during the third week of subculture. At this stage, 1 g of fresh weight (FW) was washed with dH_2O and transferred into 100 ml Erlenmeyer, and suspended in 20 ml of fresh growth media. VvSCIM13 media composition was used for *Viti's vinifera* cv. Öküzgözü (Table 2.6). The growth of the cell culture was measured by determining the fresh weight (FW), dry weight (DW), and pH value. The cell culture was harvested by vacuum filtration. The media were collected and kept at -20 °C for sugar measurement and the cells were weighed as FW. The fresh cells were dried at 85 °C in the oven for 24 h, and then DW was determined (174). The growth of *V. vinifera* cv. Öküzgözü CSC was monitored for 13 days of cultivation maintained in darkness. The cells were harvested every 2 days until day 13^{th}. After the collection of media from vacuum filtration, pH was measured for each sample by using a pH meter. Since an increase in cell water content results in an increase in fresh weight or cell size, the ratio of FW/DW was used as a measure of cell water content or cell size (259).

4.23. CELL DENSITY ANALYSIS OF *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

This study aimed to analyze the influence of initial cell density and correlation with media volume (g FW/media volume) on the growth dynamics of *V. vinifera* cv. Öküzgözü CSC.

Different concentrations of initial cells (2, 2,5, and 3 g FW cells) were transferred into 250 ml Erlenmeyer and suspended in 50 ml VlSCIM13 media based on the article (176). The subculturing was performed at 7-day and cells were measured as fresh weight. The media volume was increased from 50 ml up to 100 ml media using a 250 ml Erlenmyer, and 4,5 g (initial cell density) of cells was added. The subculturing was performed at 7-day and cells were measured as fresh weight.

4.23.1. Statistical Analysis

Correlation between initial density and media volume in *V. vinifera* cv. Öküzgözü cell suspension and comparison between the initial density of two species were analyzed. The results were analyzed statistically to determine the significant differences using Student's t-test. The null hypothesis, H0, is postulated as "initial density *does not correlate* with media volume". The alternative hypothesis, H1, is postulated as "Ho: initial density *correlates* with media volume". Type 3 (different groups with different variance) and two-tailed Student's t-test were performed with a significance level α of 0,05 and 0,01 ($p < 0,05$ and $p < 0,01$). Changed variables are species, the concentration of initial density, and the effect of media volume.

4.24. EFFECT OF SUCROSE CONCENTRATION ON CELL BIOMASS OF *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

The concentrations of sucrose in VvSCIM13 media were modified from 20 g/L to 30 g/L and a growth curve was constructed (Table 4.11). Pre-cultured 7-day-old cell suspensions of *V. vinifera* cv. Öküzgözü was filtered under vacuum and 1 g cells were transferred in 100 ml Erlenmeyer with 20 ml VvSCIM13' media in duplicate for each measurement. The Erlenmeyers in duplicate were incubated on a shaker at 110 rpm in a dark, temperature-controlled room at 25±1°C.

Table 4.11. Sucrose concentration into VvSCIM13 media composition.

Media Composition	**B5 (g/L)**	**Sucrose (g/L)**	**NAA (mg/L)**	**Kinetin (mg/L)**	**pH**

VvSCIM13	3.164	20	1	0.2	5.7
VvSCIM13'	3.164	30	1	0.2	5.7

4.25. ELICITOR TREATMENT PROCEDURE ON *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

Pre-cultured 7-day-old cell suspensions were filtered under vacuum and 1 g cells were transferred in a 100 ml Erlenmeyer with 20 ml VvSCIM13 media for *V. vinifera* cv. Öküzgözü CSC in duplicate for each measurement. The Erlenmeyers in duplicate were incubated on a shaker at 110 rpm in a dark, temperature-controlled room at 25±1°C. To stimulate t-resveratrol production from cell suspension of *V. vinifera* cv. Öküzgözü, MeJA was used as an elicitor. The MeJA was first dissolved in 96% ethanol and after the filter sterilization (45 µm syringe filter) was added into autoclaved media. MeJA was added at the beginning of the log phase (on the 4th day) at a concentration of 100 µM (124) (Table 4.12).

Table 4.12. Elicitor treatment in *V. vinifera* cv. Öküzgözü CSC.

Days of Fermentation	Elicitors / TIME (sampling)	Control	MeJA (100µM)
1	Day 1	C1, C2	M1, M2
2			
3	Day 3	C1, C2	M1, M2
4			MeJA was added
5	Day 5	C1,C2	M1,M2
7	Day 7	C1,C2, C3	M1,M2
9	Day 9	C1, C2	M1, M2
11	Day 11	C1, C2	M1,M2
13	Day 13	C1, C2	M1,M2

4.25.1. Effect of MeJA on cell biomass of *V. vinifera* cv. Öküzgözü cell suspension culture

The effect of the MeJA on cell growth of *V. vinifera* cv. Öküzgözü CSC was checked by determining the cell dry weight with the same procedure of dry weight determination used

for control samples. MeJA was added to the suspension culture at the beginning of their log phase of growth (at 4th day).

4.26. CELL VIABILITY TEST OF *V. VINIFERA* CV. ÖKÜZGÖZÜ CELLS WITH FLUORESCENCE MICROSCOPY

The Fluorescein diacetate test (FDA) was used for cell viability measurement. Twenty-five milligrams of FDA were dissolved with 5 ml acetone. In a laminar flow, 950 µL of the sample (7 – day-old cells) was transferred from continuous *V. vinifera* cv. Öküzgözü CSC to 2 ml Eppendorf tubes. One ml of VvSCIM13 media was added in 2 ml Eppendorf tubes that contain cell suspensions. Then, 50 µL of FDA was added into 2 ml Eppendorf. Eppendorf tubes were covered with aluminum foil and incubated for 5 minutes at room temperature. FDA is digested by esterases within living cells, releasing the fluorescein molecule, which lights up under UV light. After 5 minutes, a drop of the sample was taken and put into the microscope slide. A cover slide was used to cover the slide. Living cells were detected by using a fluorescence microscope (202).

4.27. EXTRA-CELLULAR TRANS-RESVERATROL EXTRACTION FROM *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

Five milliliters of the sample were mixed with 5 mL of 100% ethyl acetate and mixed for 5 min to extract extracellular t-resveratrol. The mixture was allowed to settle for 15 minutes at room temperature then the upper phase was collected. The removed lower phase was re-extracted by adding 5 ml ethyl acetate two times. The ethyl acetate was removed from the organic phase by rotary evaporation at 35 °C for around 3h. The pellet was resuspended in 1ml MeOH and kept at -20°C for HPLC analysis. All procedure was performed in dark (174) (124) (260) (Figure 4.7).

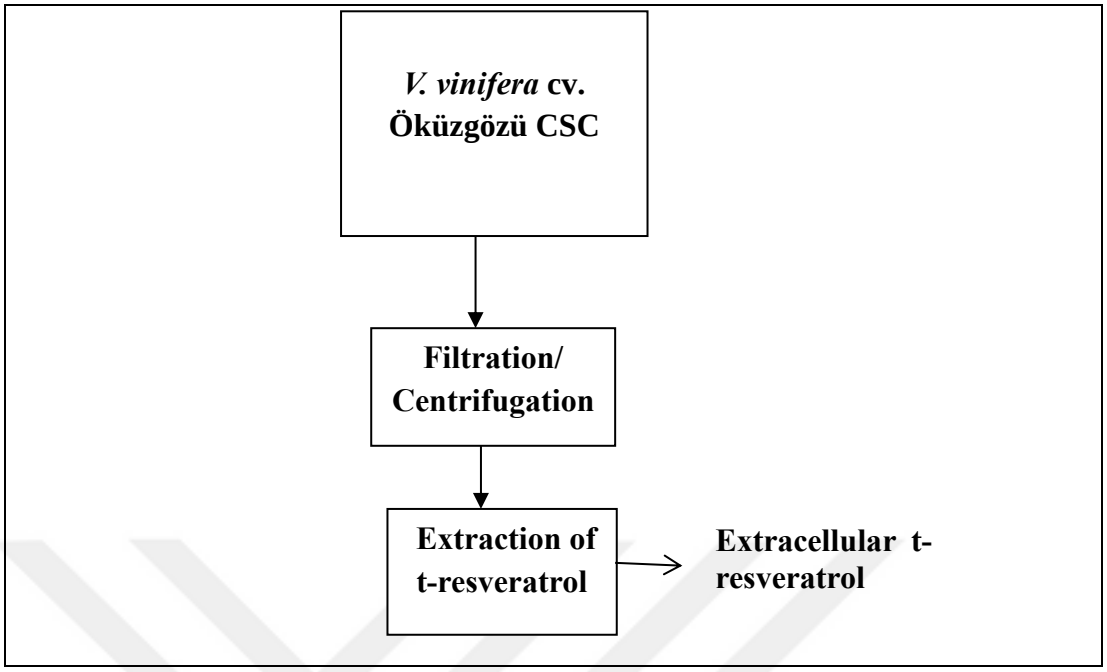


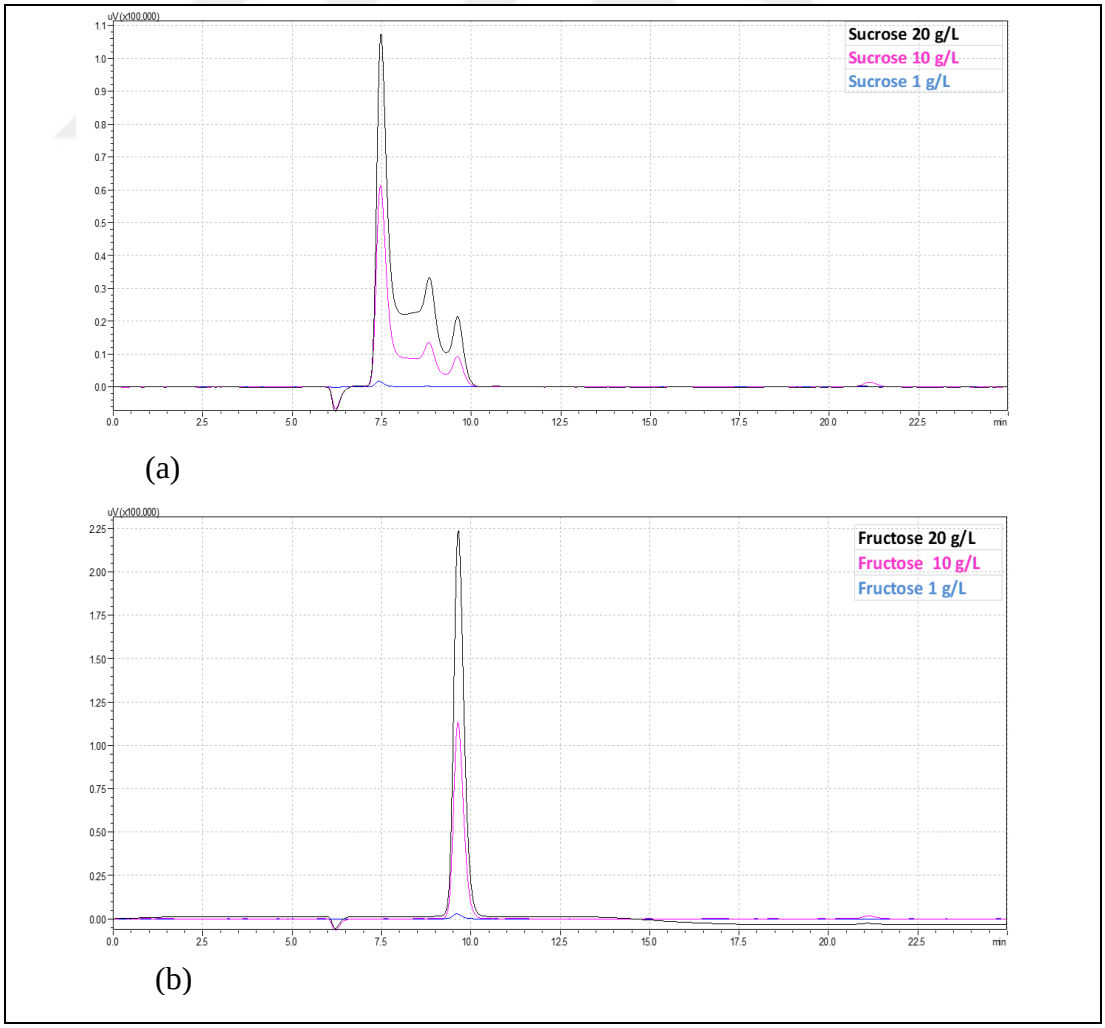
Figure 4.7. Downstream process for extracellular t- resveratrol production (261)

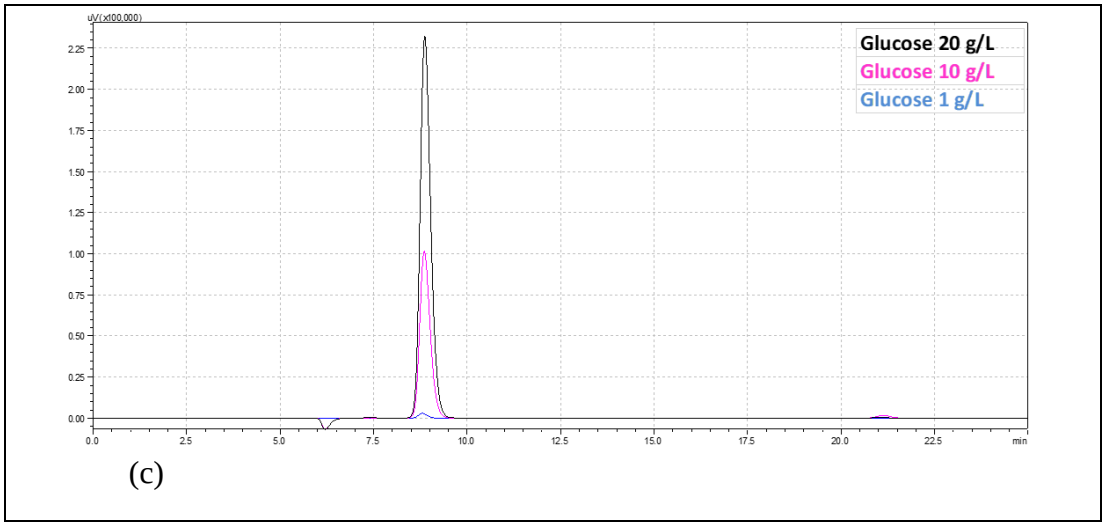
4.27.1. HPLC analysis of extra-cellular t-resveratrol production from *V. vinifera* cv. Öküzgözü cell suspension culture

The t-resveratrol analysis was performed on HPLC (Column: Agilent ZORBAX Eclipse XDB columns C18) according to the procedure from Zamboni et.al, (2006) (176). Owing to the column diameters used in this article being different from the own column we used, the injection rate and flow rate were changed. The flow rate was 0.7 ml/min and the injection volume was 25 µl. The UV-VIS spectra were recorded at 310 nm. The mobile phases consisted of 0.1 % acetic acid in H_2O (A) and acetonitrile (B). All mobile phase was filtered through 0.22 µm RC (Regenerated cellulose) membrane to remove particulate matter. Separation was carried out at 40 °C in 30 min, under the following conditions: linear gradients starting at 5% B, to 70 % B in 25 min, to 95 % B in 0.1 min, 95 % B for 2 min, back to 5 % B in 0.1 min. The concentrations of resveratrol were prepared to a range from 0.1-10 mg/L by dissolving in MeOH (HPLC grade). The standards and samples were stored at -20 °C until HPLC analysis.

4.28. SUGAR ANALYSIS FOR *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

The residual sugar contents in *V. vinifera* cv. Öküzgözü suspension medium was measured by the HPLC (LC Shimadzu) using Aminex HPX-87H HPLC, 300 x 7.8 mm column by refractive index detector, at a working flow rate of 0.6 ml/min at 50°C using 5 mM H_2SO_4 as the mobile phase. The samples were filtered through a 0.22 µm RC membrane filter. Each sample was injected twice with a 20 µL injection volume and the peak was detected. Different concentrations (20, 10, 1.0, 0.5, 0.25, and 0.1 g/L) of sucrose, glucose, and fructose were used for standard curve construction. The remaining concentrations of sucrose, glucose, and fructose in the samples were identified by comparing their counted peak area with the standard curves. The retention times for sucrose, glucose, and fructose were 7.412, 8.801, and 9.598 minutes, respectively (Figure 4.8).





(c)

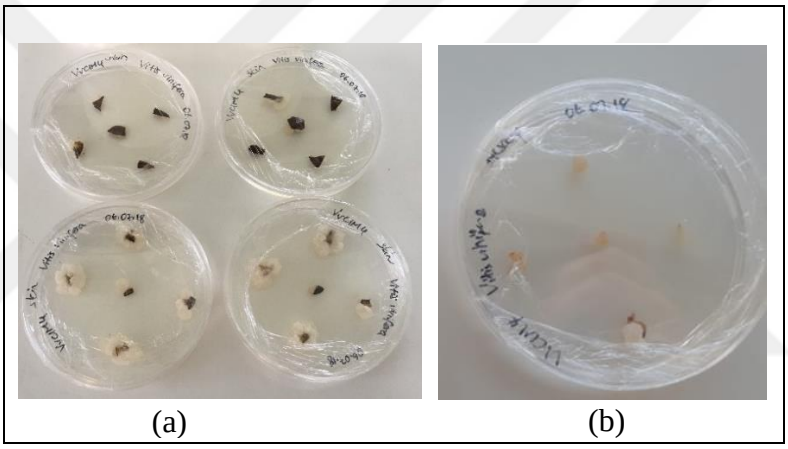
Figure 4.8. Chromatograms of sugar standards. (a) Sucrose, (b) Fructose, and (c) Glucose for *V. vinifera* cv. Öküzgözü CSC

5. RESULTS AND DISCUSSION

V. LABRUSCA (L.) CV. ISABEL

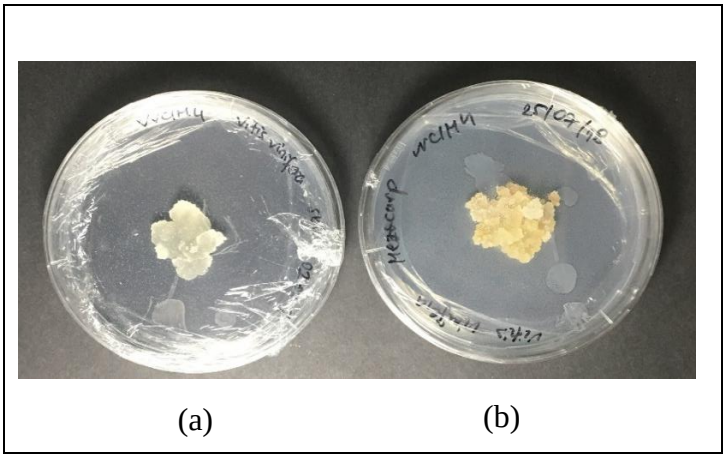
5.1. CALLUS INDUCTION OF *V. LABRUSCA* CV. ISABEL

After 4 weeks, the different callus formation was shown from the skin and mesocarp explant and it was separated and maintained at 25°C in the dark and light and sub-cultivated on a solid growth medium every two weeks (Figure 5.1 a,b, and Figure 5.2 a,b).



(a) (b)

Figure 5.1. Callus induction from *V. labrusca* cv. Isabel. (a) callus formation from skin explant and cultivated in VICIM4 media, and (b) callus formation from mesocarp explant and cultivated in VICIM4 media



(a) (b)

Figure 5.2. Callus of *V. labrusca* cv. Isabel. (a) from the skin, and (b) from mesocarp cultivated in the dark in VICIM4 media

We have observed the differences in the color of *V. labrusca* cv. Isabel calli were placed in dark and light conditions. *V.labrusca* cv. Isabel calli cultivated in the light turned in red whereas others grown in the dark remained as yellow (Figure 5.3 a, b).

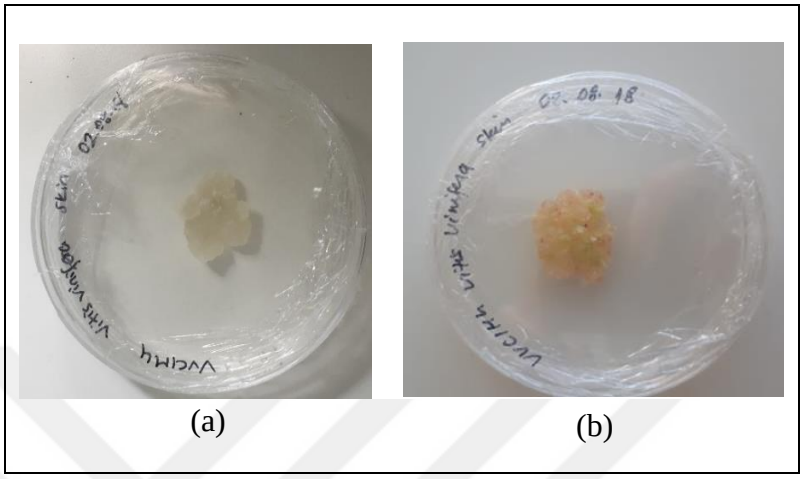


Figure 5.3. Callus of *V. labrusca* (L.) cv. Isabel from skin cultivated in (a) dark, and (b) light conditions in VICIM4 media

Repeated selection of the tissues by eliminating the necrotic part and with the healthy tissues subculturing was performed to get cell lines of *V. labrusca* cv. Isabel (Figure 5.4 a,b).

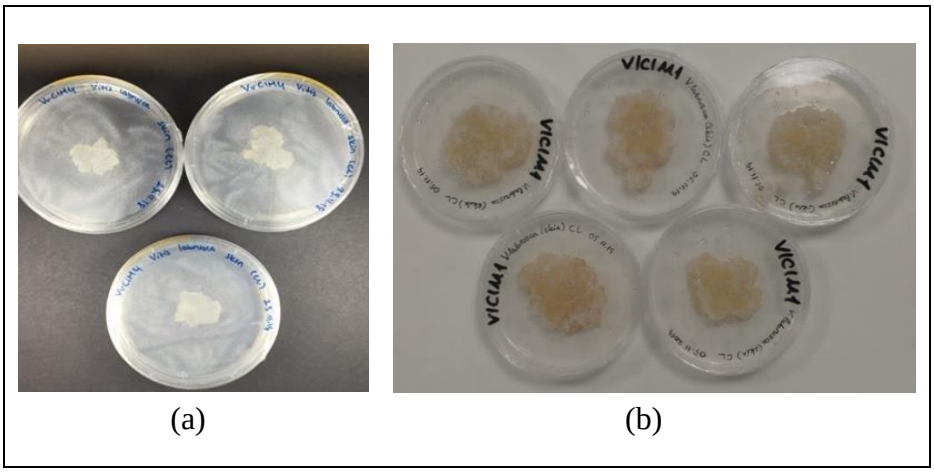


Figure 5.4. Cell lines of *V. labrusca* (L.) cv. Isabel. (a) VICIM4 media (5 months old), and (b) in VlCIM1 (14 months old)

5.2. ESTABLISHMENT OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

V. labrusca cv. Isabel CSC cultivated into VlSCIM6 media and maintained in the dark conditions remained yellow, whereas the cells maintained in the light conditions turned in brown (Figure 5.5a,b).

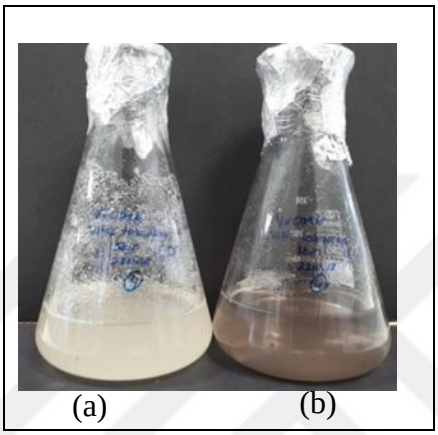


Figure 5.5. *V. labrusca* cv. Isabel CSC growth (a) in the dark (VlSCIM6) media, and (b) in the light (VlSCIM6) media

CSC of *V. labrusca* cv Isabel grown into VlSCIM2 media have shown the best performance of cells growing (Figure 5.6a and 5.7a, and Figure 5.8a,b). In *V. labrusca* cv. Isabel, the CH presence in media shows better results on cell biomass growth (Figure 5.6-5.8). Also, it was studied the effect of MS medium using, but as it is shown through the pictures, MS medium did not work for *V. labrusca* cv. Isabel CSC (Figure 5.7 c,d).

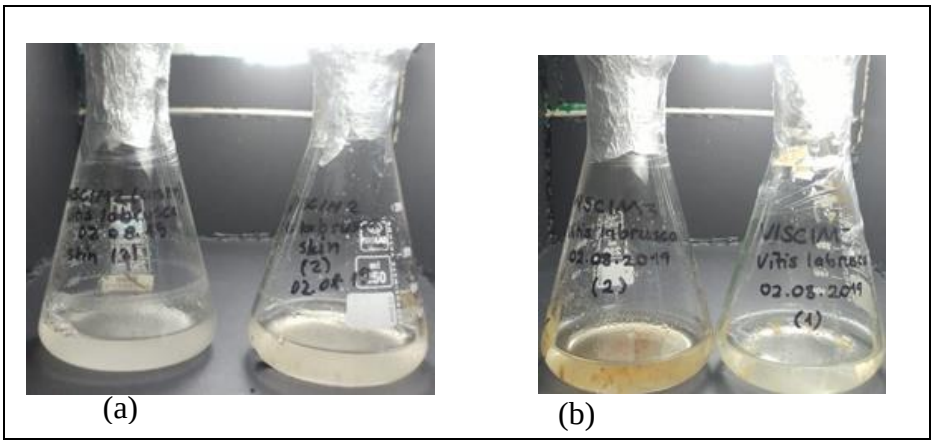


Figure 5.6. *V. labrusca* cv. Isabel CSC into (a) VlSCIM2, and (b) VlSCIM3 media

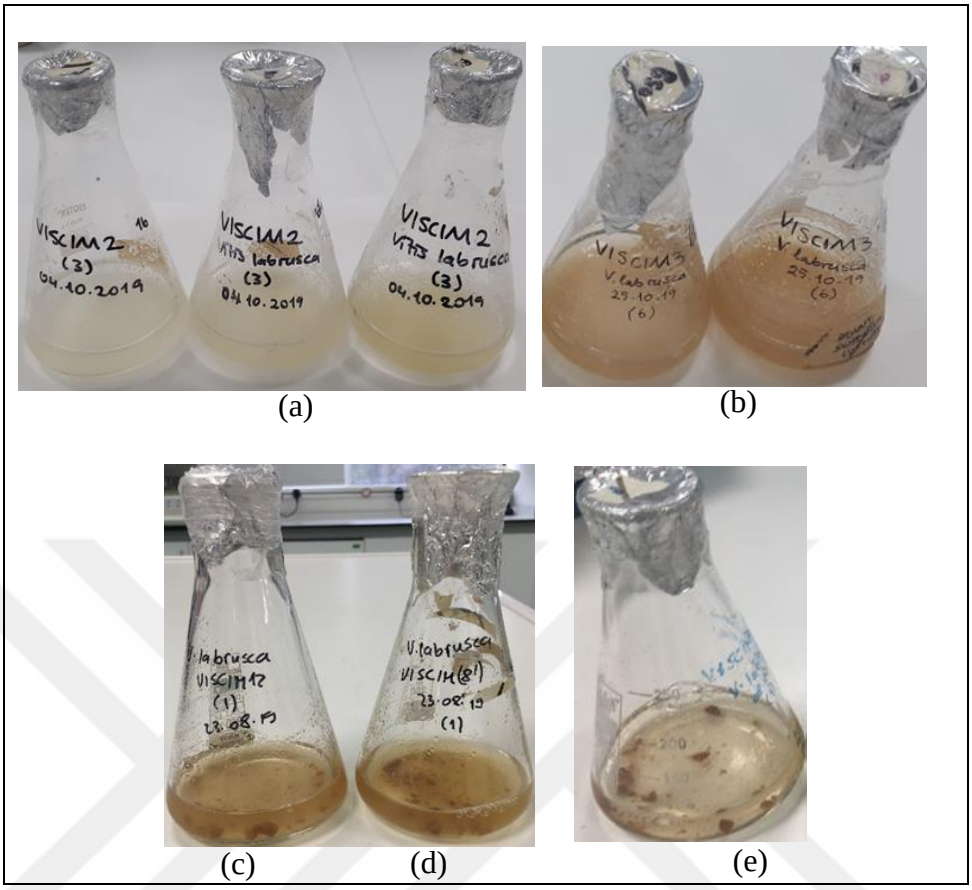


Figure 5.7. *V. labrusca* cv. Isabel CSC into (a)VlSCIM2, (b) VlSCIM3, (c)VlSCIM12, (d)VlSCIM (8'), and (e) VlSCIM7

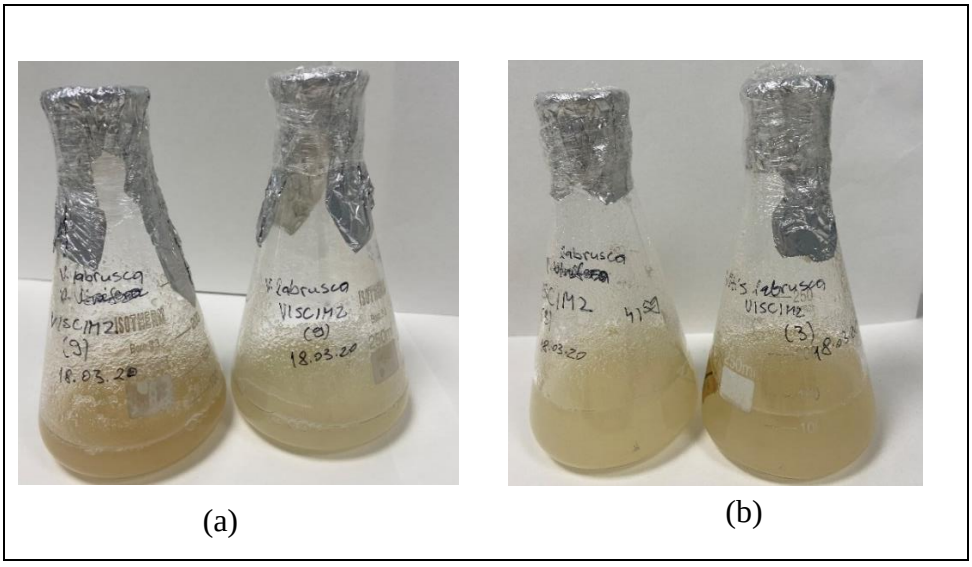


Figure 5.8. *V. labrusca* cv. Isabel CSC into (a) 50 ml VlSCIM2 media, and (b) 100 ml VlSCIM2 media

5.3. DETERMINATION OF CELL GROWTH (FW AND DW) AND PH VALUE FOR *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

First study

Using VlSCIM2 media, *V. labrusca* cv. Isabel cells showed an exponential phase between days 5 and 11, followed by a stationary phase until day 13 (Figure 5.9). According to the results, the FW on 1st day was 38.96 g/L, whereas, on the 11th day was 487.08 g/L. On the other hand, DW increased from 1.34 g/on the 1st day up to 11.65 g/L on the 13th day (Figure 5.9). The ratio of FW/DW increased as sucrose concentration dropped in media as was shown in the graphs (Figure 5.9 b). The doubling time of *V. labrusca* cv. Isabel's cells were 6.9 days. The maximum specific growth rate for *V. labrusca* cv. Isabel's cells were 0.35/day (Figure 5.9 c).

Using VlSCIM2 media, the pH profile during the lag period initially decreases (pH=5.47) due to the acidification of media because of the cell lysis and the initial ammonium uptake, and during the exponential growth phase (on the 11th day) it increases (up to pH=6.81). Then, pH achieved a stable level which is correlated to the nitrate uptake (Figure 5.9 d) (266).

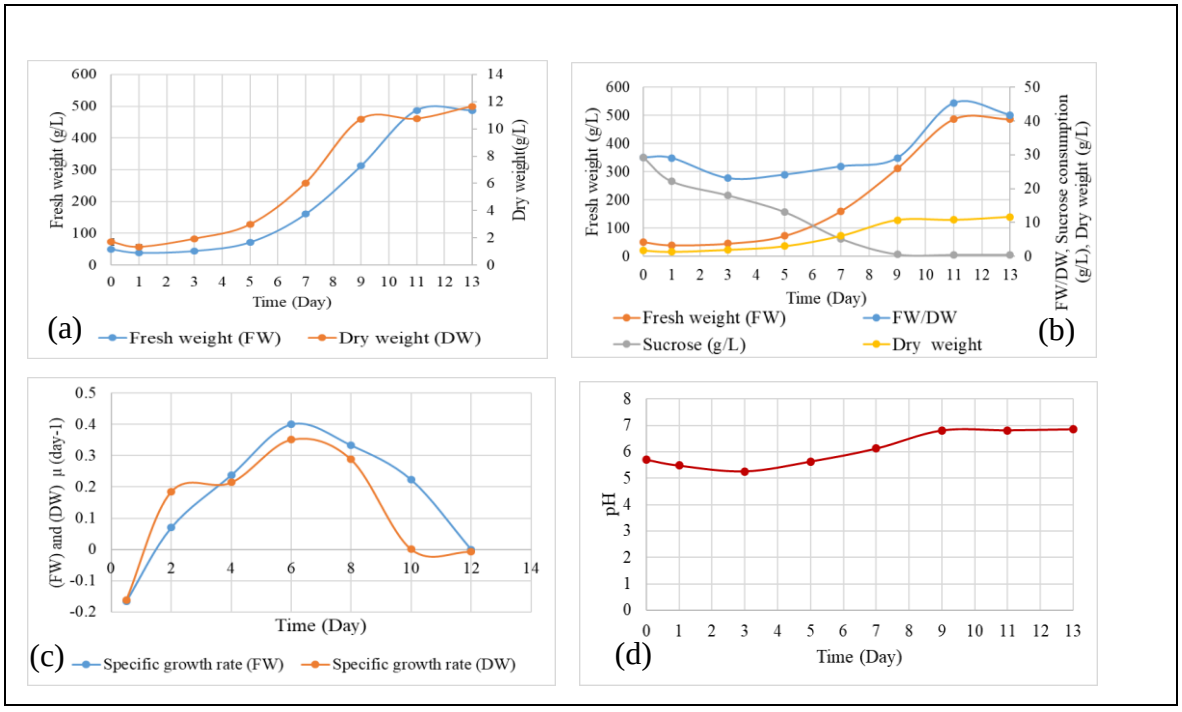


Figure 5.9. Growth profile of *V. labrusca* cv. Isabel CSC. (a) FW, DW, (b) ratio between FW/DW and sugar consumption, and (c) Specific growth rate (μ), (d) pH profile. Standard deviations for fresh weight were found to be between 0.03-0.2. Standard deviations for dry

weight were found to be between 0.03-0.2. Standard deviations for pH were found to be between 0.01-0.05

Second study

Using VlSCIM2 media, *V. labrusca* cv. Isabel cells showed an exponential phase between days 5 and 15, followed by a stationary phase until day 19 (Figure 5.10 a). According to the results, the FW on day 0 was 50 g/L whereas, on the 15th day was 614.32 g/L. On the other hand, the DW increased from 2.05 g/ on day 0 up to 14.57 g/L on the 11th day. The maximum specific growth rate for *V. labrusca* cv. Isabel's cells were 0.47/day (Figure 5.10b).
Using VlSCIM2 media, the pH profile during the lag phase initially decreases (pH=5.38), and an increasing trend is shown during the exponential growth phase (up to pH=7.37) on the 9th day. A lower value of pH happens due to the acidification of media because of the cell lysis and the initial ammonium uptake. Then, pH increases at day 21, and after that pH achieved a stable level which is correlated to the nitrate uptake (Figure 5.10 a) (266).

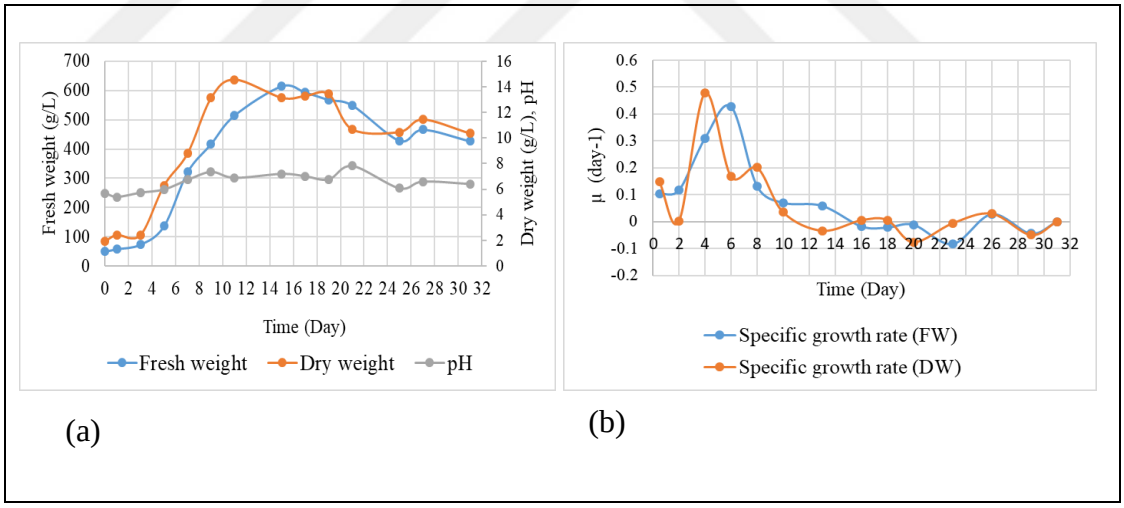


Figure 5.10. Determination of cell growth for *V. labrusca* cv. Isabel CSC. (a) FW, DW, pH, and (b) Specific growth rate (μ). Standard deviations for fresh weight were found to be between 0.01-0.2. Standard deviations for dry weight were found to be between 0.0009-0.15. Standard deviations for pH were found to be between 0.002-0.02

Third study

Using VlSCIM3 media, *V. labrusca* cv. Isabel cells showed an exponential phase between days 3 and 9, followed by a stationary phase until day 15 (Figure 5.11 a). According to the

results, the FW at 0 days was 50 g/L whereas on the 15th day was 567.12 g/L. On the other hand, the DW increased from 1.925 g/L at 0 days up to 14.48 g/L on the 9th day. The maximum specific growth rate for *V. labrusca* cv. Isabel's cells were 0.36/day (Figure 5.11 b).

Using VlSCIM3 media, the pH profile during the lag phase increases (pH=6.1), and an increasing trend is shown during the exponential growth phase (up to pH=7.37) on the 9th day. A lower value of pH happens on days 9 and 11 due to the acidification of media because of the cell lysis and the initial ammonium uptake. Then, pH increases and achieved a stable level which is correlated to the nitrates uptake. (Figure 5.11a) (266).

The biomass concentration reached in control cells of *V. labrusca* cv. Isabel (including three studies) is higher than in other studies using different varieties of *V. vinifera* (L): 11 g DW/L (133), 10.9 g DW/L (124). A comparable result of biomass (14.57 g/L DW) was reached with another study that used another variety of *V. Labrusca* (*Vitis labruscana* L. cv. Campbell Early) (16 g/L DW) (267).

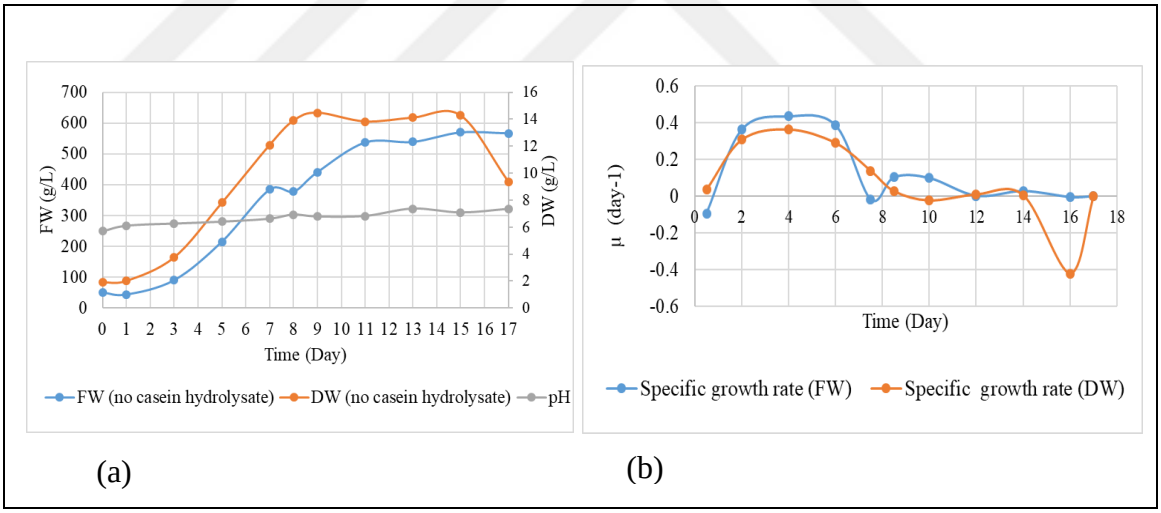


Figure 5.11. Determination of cell growth for *Vitis labrusca* cv. Isabel CSC. (a) FW, DW, (b) Specific growth rate (μ), and (c) pH profile. Standard deviations for fresh weight were found to be between 0.005-0.16. Standard deviations for dry weight were found to be between 0.0009-0.19. Standard deviations for pH were found to be between 0.0009-0.01

5.4. CELL DENSITY ANALYSIS OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

First study

Between 2 g and 2.5 g of initial density added on 50 ml media, the FW measured on day 7 showed no significant difference at levels α=0,05 and α=0.01 (Figure 5.12 a).

Between 2, 2,5 g, and 3.1 g of initial density added on 50 ml media, the FW measured on day 7 showed a significant difference at level α=0,05 (Figure 5.12 a).

The results of FW between 50 ml and 100 ml media volume showed no significant difference at levels α=0,05 and α=0.01 (Figure 5.12 a).

Between the initial FW (2 g) and final FW on day 7 was found significant difference at level α=0,05 (Figure 5.12 a).

Between the initial FW (2.5 g) and final FW on day 7 was not found a significant difference at levels α=0,05 and α=0,01 (Figure 5.12 a).

Between the initial FW (4.5 g) and final FW on day 7 was not found a significant difference at levels α=0,05 and α=0,01 (Figure 5.12 b).

Between the initial FW (9 g) and final FW on day 7 was no found significant difference at level α=0,05 (Figure 5.12 c).

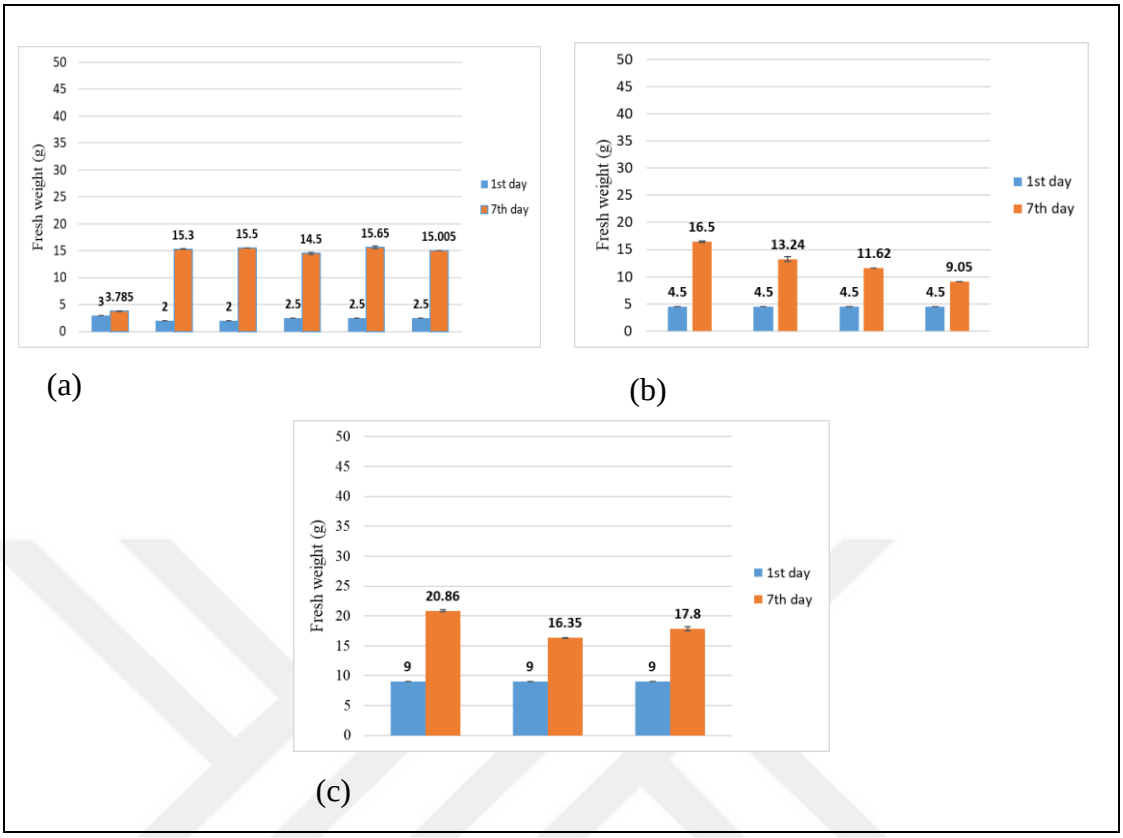


Figure 5.12. Cell density analysis in *V. labrusca* cv. Isabel CSC. (a) 50 ml media/250 ml Erlenmeyer, (b)100 ml media/250 ml Erlenmeyer, and (c) 200 ml media/500 ml Erlenmeyer (First study)

Second study

The synergistic effect of combined initial inoculum density with the volume of media in flasks in *V. labrusca* cv. Isabel CSC is shown in Figure 5.13. The final FW concentration in the 100 ml media/250 ml Erlenmyer was double that of the final FW concentration in the 50 ml media/250 ml Flask (Figure 5.13 a,b). Further, an increasing media volume and an initial inoculum density increased the final FW concentration (Figure 5.13 c). There are many articles on the effect of the initial inoculum/volume of the media on biomass and secondary metabolite production (244) (252)(268)(269)(270). In the suspension culture of *Perilla frutescens*, maximum biomass of 38.3 g DW/L was achieved at an inoculum size of 50 g DW/L, and anthocyanin production was enhanced 23-fold. In the CSC of *Gymnema sylvestre*, the different amounts of inoculums (2.5, 5.0, 10.0, and 20.0 g/L) were tested, the optimum density of biomass (11.25 g/L), as well as gymnemic acid (9.95 mg/g DW), was achieved with 10.0 g/L inoculum.

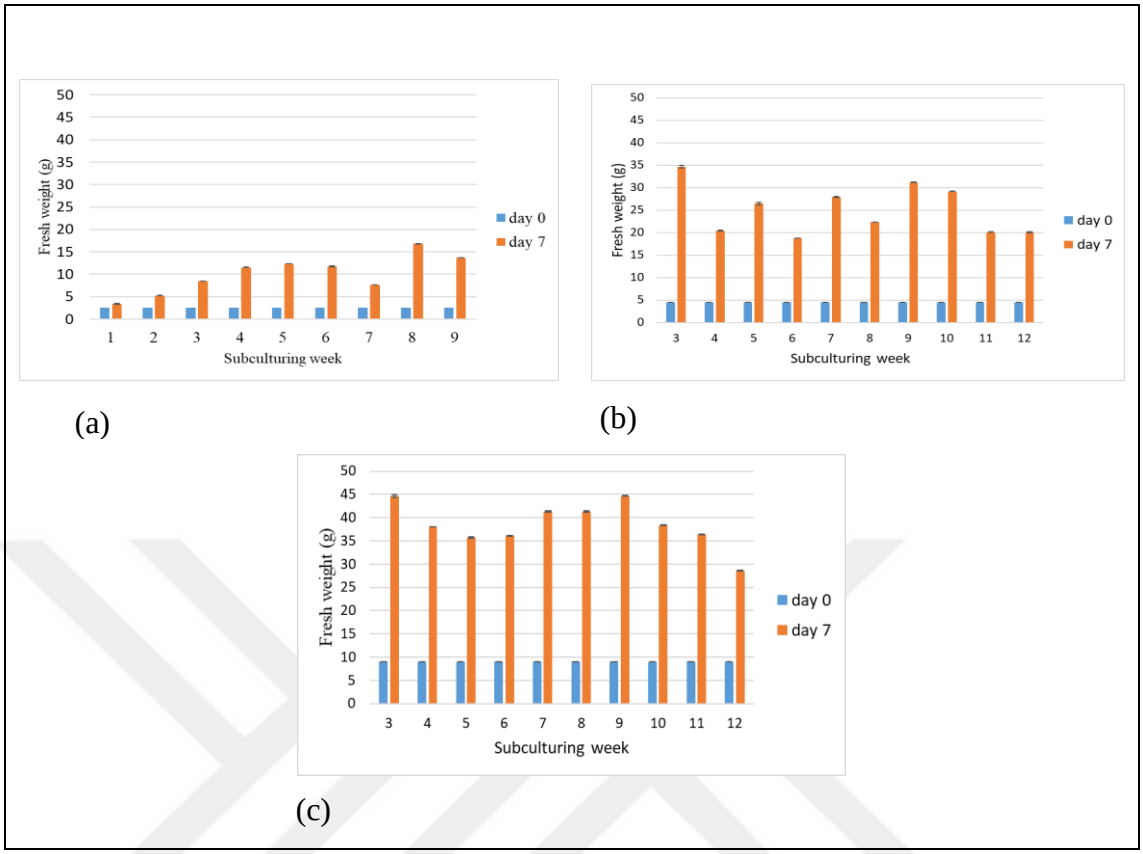
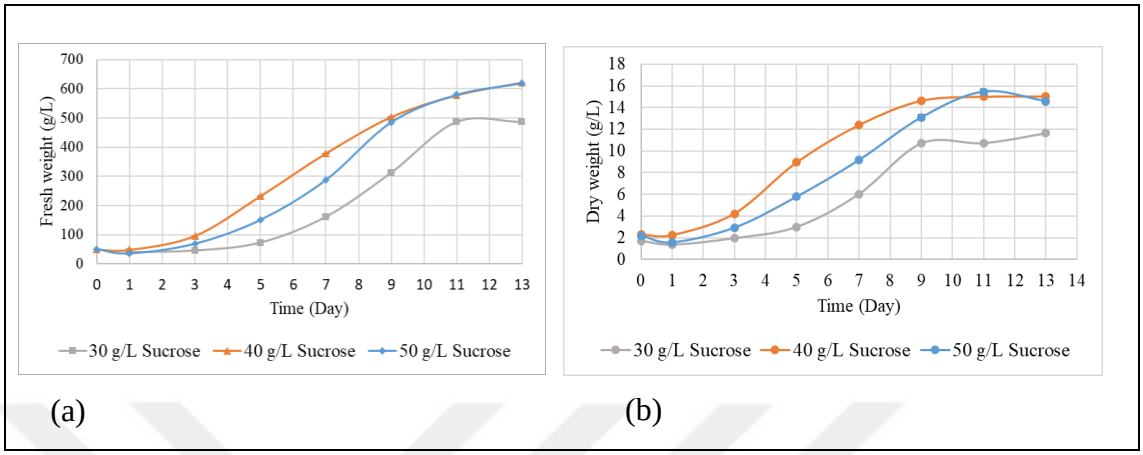


Figure 5.13. Cell density analysis in *V. labrusca* cv. Isabel CSC. (a) 2.5 g FW/50 ml media, (b) 4.5 g FW/100 ml media, and (c) 9 g FW/200 ml media (Second study)

5.5. EFFECT OF SUCROSE CONCENTRATION ON CELL BIOMASS OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

To optimize the concentration of sucrose, it was used at 3 % -5 % concentration in *Vitis labrusca* cv. Isabel CSC. The *V.labrusca* cv. Isabel cells supplemented with 3% sucrose showed an exponential growth phase between day 3 and 11 followed by a stationary phase up to day 13, while the cells grown with 4% and 5% sucrose, displayed an exponential phase from day 3 and did not reach the stationary growth phase. 3% sucrose added to the culture resulted in lower FW and DW of cells (Figure 5.14 a,b). On the other hand, cells grown in media with 4% sucrose produced higher FW and DW of cells (Figure 5.14 a, b). However, when the sucrose level was further increased (5%) (Figure 5.14 a, b), there was a decreasing tendency in cell growth. This was attributed to the inhibition of nutrient uptake caused by an increase in the osmotic potential and the high viscosity of the medium (271). A similar result was reported by See KS et al, 2010 (271) that supplementing the culture medium with 4.5%

and 6% sucrose showed a decreasing line in cell growth of *Melastoma malabathricum L.* CSC.



(a) (b)

Figure 5.14. Sucrose effect on the biomass of *V. labrusca* cv. Isabel CSC. (a) FW, (b) DW. 30 g/L sucrose (standard deviation was found between 0.03 to 0.2), 40 g/L sucrose (standard deviation was found between 0.008 to 0.2), and (c) 50 g/L sucrose (standard deviation was found between 0.05 to 0.2)

5.6. ELICITOR TREATMENT PROCEDURE ON *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

The effect of the MeJA on cell growth of *V. labrusca* cv. Isabel CSC was checked twice by cell FW and DW determination for the thirteenth day using the same procedure FW and DW determination performed for control samples. In flask experiments, elicitation by MeJA induced a change of cell color from yellow to dark brown, and cell growth was inhibited with MeJA presence compared with the non-treated control samples.

As shown in Figure 5.15 a, b, the FW and DW of treated cells with MeJA decreased (148 g/L FW and 254 g/L FW; 6 g/L DW and 10 g/L DW) based on the FW and DW of cells achieved in control samples (SCC (487 g/L FW and 514 g/L FW; 11.65 g/L DW and 14.57 g/ L DW). This shows, that after elicitation some cells died so the FW and DW of cells decreased (133) (126). On the other hand, the pH profile during the lag period initially decreases (pH=5.63), while it increases before adding the MeJA (pH=6.44). After MeJA was added, the pH started to decrease (Figure 5.15 b). Resveratrol stimulated its production and it stopped the growth of cells because cell division metabolism is antagonistic to its production. By acting as a defender, resveratrol cause cell death (126)(133)(258). The effect

of MeJA on cell growth has been reported in many articles (133) (137) (169) (272) and this effect has been suggested to be caused by the diversion of the carbon allocation, favoring the activation of the secondary metabolism over the primary metabolism (133). Cell growth affected in elicitor-treated cells was showed also by cell viability measured by fluorescence microscopy of cells treated with FDA (Figure 5.17).

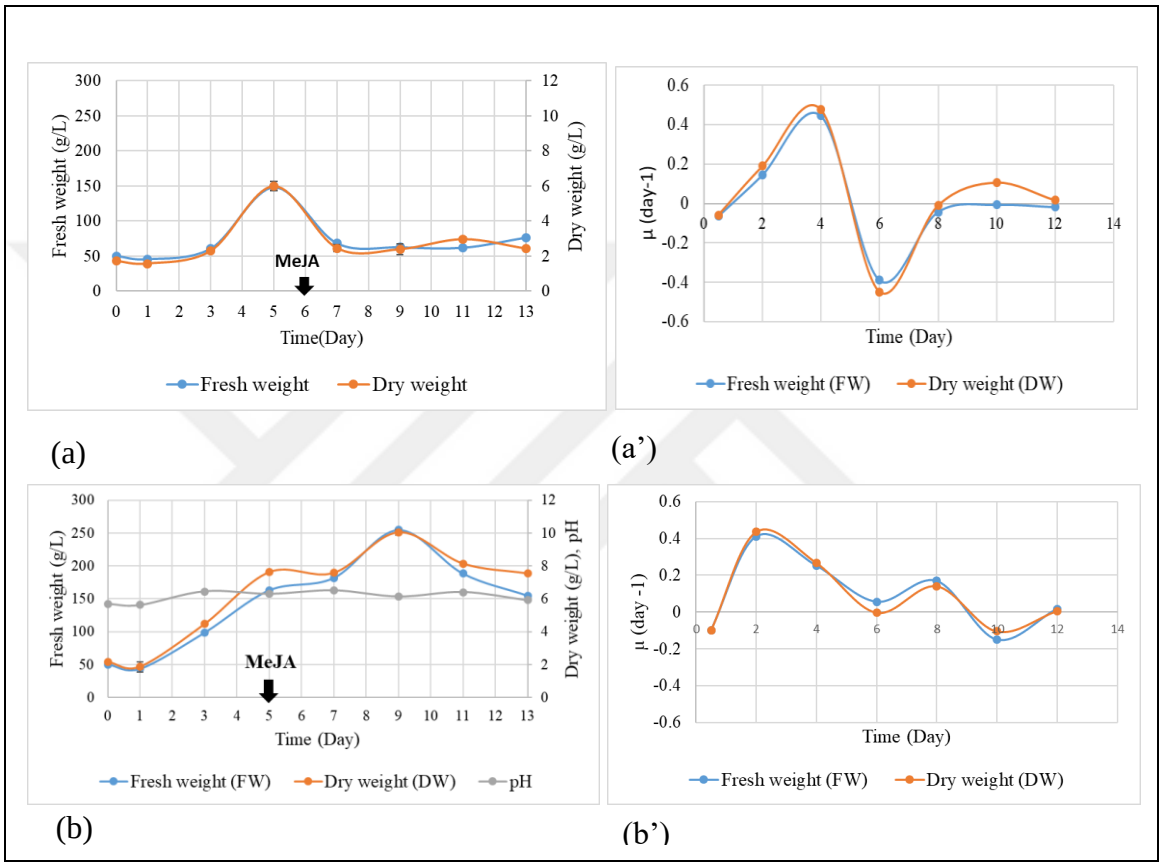


Figure 5.15. Effect of MeJA on cell biomass of *V. labrusca* cv. Isabel CSC. (a) FW, DW (First study), (a') Specific growth rate, (b) FW, DW, pH (Second study), and (b') Specific growth rate

Second study

Figure 5.16 shows the FW of treated cells with MeJA, ß-CDs, and a combination between MeJA and ß-CDs decreased based on the FW reached in control cells. This shows, that after elicitation some cells died so the FW of cells decreased (133) (126). Cell division metabolism is antagonistic with t- resveratrol production, thus, it triggers its production and inhibits living cells' growth. By acting as a defender, resveratrol cause cell death (258). Similar results were reported by Lijavetzky D. et al (137) and Belchi-Navarro. S. et al (133)

where cells treated with MeJA and a combination of MeJA with ß-CDs have shown lower biomass production compared with biomass from control cells. On the other hand, the article Xu. et al (186) have shown the opposite where no significant decline in biomass production using 100 µM MeJA, but MeJA at 150 and 200 µM caused a significant decline of DW.

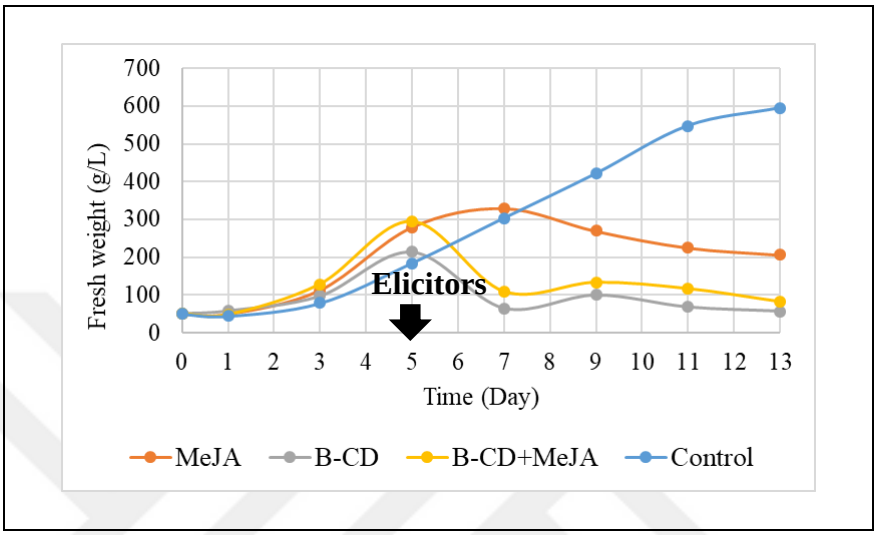


Figure 5.16. Growth profile of *Vitis labrusca* cv. Isabel before and after elicitation

5.7. CELL VIABILITY TEST OF *VITIS LABRUSCA* CV. ISABEL CELLS WITH FLUORESCENCE MICROSCOPY

Cells treated with MeJA stopped growing. In comparison to control cells, the fluorescence microscopy measurement of cell viability in cells treated with FDA also appears to be impacted (Figure 5.17 a, b).

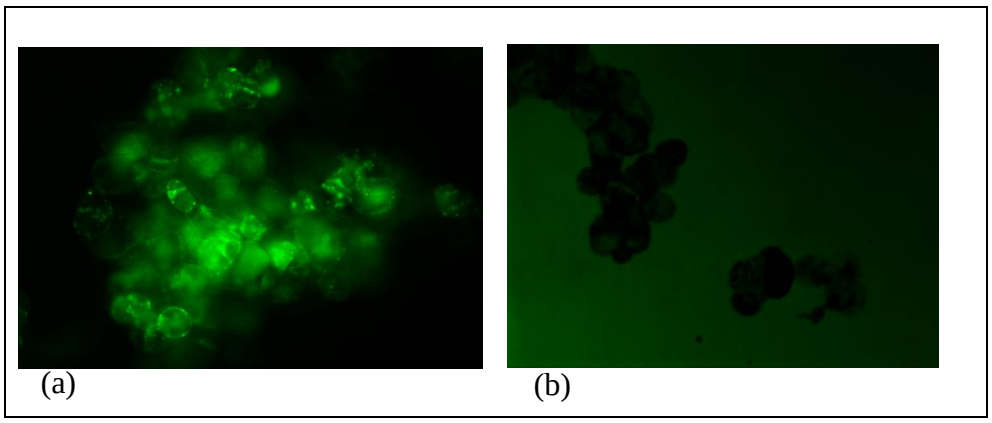


Figure 5.17. *V.labrusca* cv. Isabel cell viability (a) before elicitation, and (b) after elicitation

5.8. EXTRACELLULAR TRANS-RESVERATROL PRODUCTION FROM *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

First study

Extracellular t-resveratrol was identified and quantified by comparison with the authentic standard of 98 % purity resveratrol based on its retention time. Under these HPLC conditions, the retention time of resveratrol was 16.4922 min. The calibration curve was obtained from the increases in the peak areas of standard t-resveratrol which was observed at minutes 16.4922 min (Figure 5.18). The X-axis intercepts were taken into account for t-resveratrol amount determination. The calibration curve of standard t-resveratrol is shown in Figure 5.19.

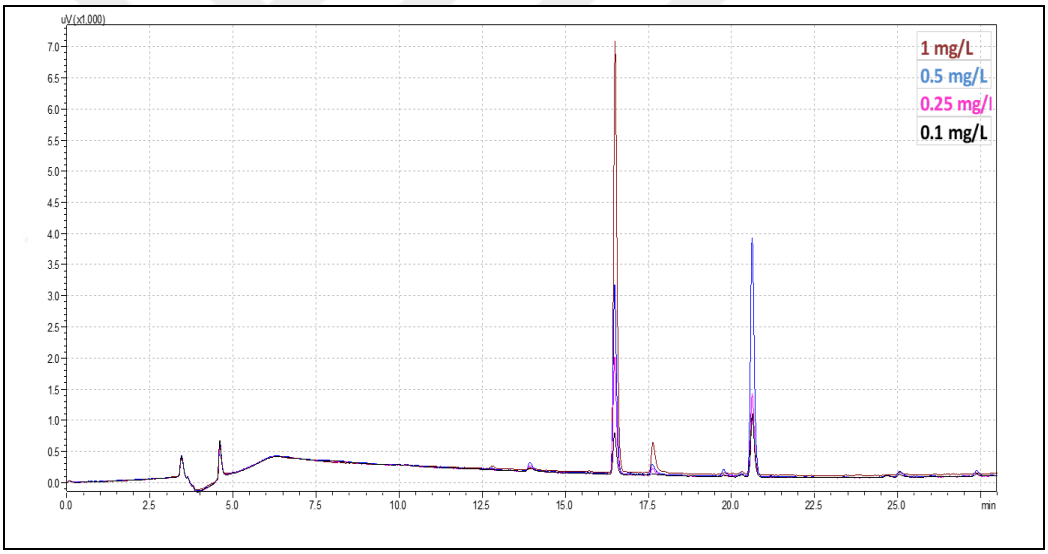


Figure 5.18. HPLC chromatogram of standard t-resveratrol for *V.labrusca* cv. Isabel (First study)

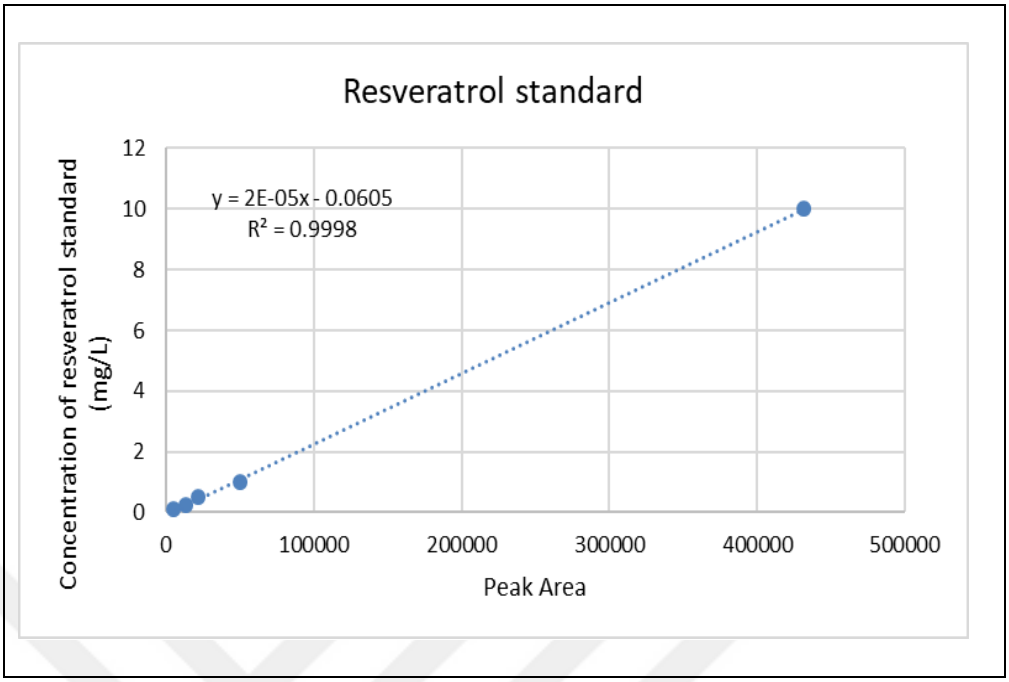


Figure 5.19. The calibration curve of standard t-resveratrol for *V.labrusca* cv. Isabel (First study)

t-resveratrol production was monitored in *V. labrusca* cv. Isabel CSC before and after supplying MeJA on CSC. In *V. labrusca* cv. Isabel CSC, t-resveratrol was monitored also after supplying MeJA + ß-CD elicitors. Figure 5.20 represents an HPLC chromatogram showing the peaks of extracellular t-resveratrol production in *V. labrusca* cv. Isabel.

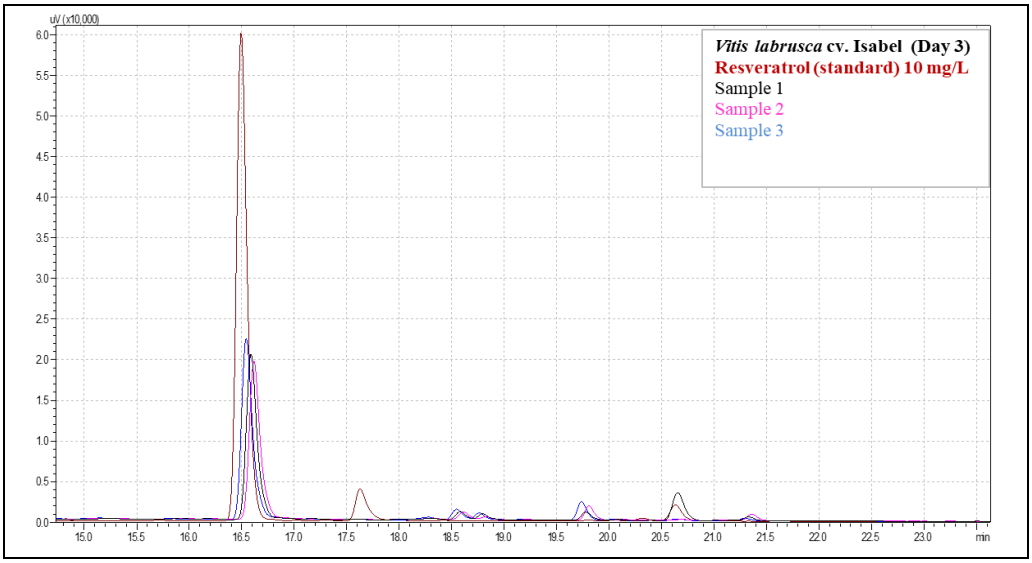


Figure 5.20. HPLC chromatograms of extracellular t-resveratrol from *V. labrusca* cv. Isabel cells (Day 3 (triplicate) - before elicitation with MeJA)

The production of t-resveratrol in *Viti's* varieties of cell culture with different elicitors has been studied by several articles as Table 2.4 showed.

Unlike the results performed in many articles where they have shown no resveratrol production without elicitation, this thesis shows the opposite. In control samples of *Vitis labrusca* cv. Isabel, the t-resveratrol was found on day 1 (0.43 mg/L) and day 3 (0.91 mg/L). After day 3, no resveratrol was found in control samples of *V. labrusca* cv. Isabel CSC. Figure 5.21 shows the effect of MeJA and ß-cyclodextrin concentration on t-resveratrol production in *V. labrusca* cv. Isabel CSC. 3.73 mg/L of t-resveratrol was found on day 3 and decreased drastically on day 5 (before adding MeJA). Adding MeJA on day 6 stimulated the level of t-resveratrol to increase, but after the 7th day was detected lower t-resveratrol. In grapevine CSC, MeJA changes the pattern of cell growth in addition to inducing resveratrol production. Cells of *V. labrusca* cv. Isabel were treated also with ß-CD on 1st day and the MeJA was added to cells five days later of cell culturing as shown in Table 4.4. On day 1, in the presence of ß-CD, 0.37 mg/L t-resveratrol was found, whereas no t-resveratrol was found in the presence of ß-CD and MeJA (Figure 5.21).

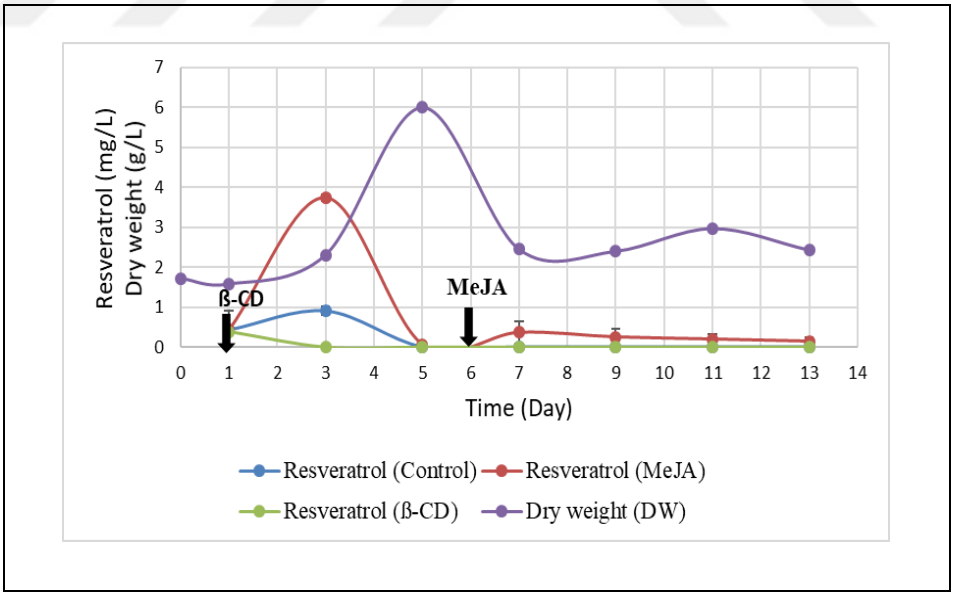


Figure 5.21. Extracellular t-resveratrol production *V. labrusca* cv. Isabel CSC. Control samples and samples treated with MeJA (100µM), ß-CDs (50Mm), and in combination between MeJA and ß-CDs (50mM ß-CD+100µM MeJA)

Second study

Extracellular t-resveratrol was identified and quantified by comparison with the authentic standard of 98 % purity resveratrol based on its retention time. Under these HPLC conditions, the retention time of resveratrol was 16.745 min. The calibration curve was obtained from the increases in the peak areas of standard t-resveratrol which was observed at minutes 16.745 min (Figure 5.22). The X-axis intercepts were taken into account for t-resveratrol amount determination. The calibration curve of standard t-resveratrol is shown in Figure 5.23.

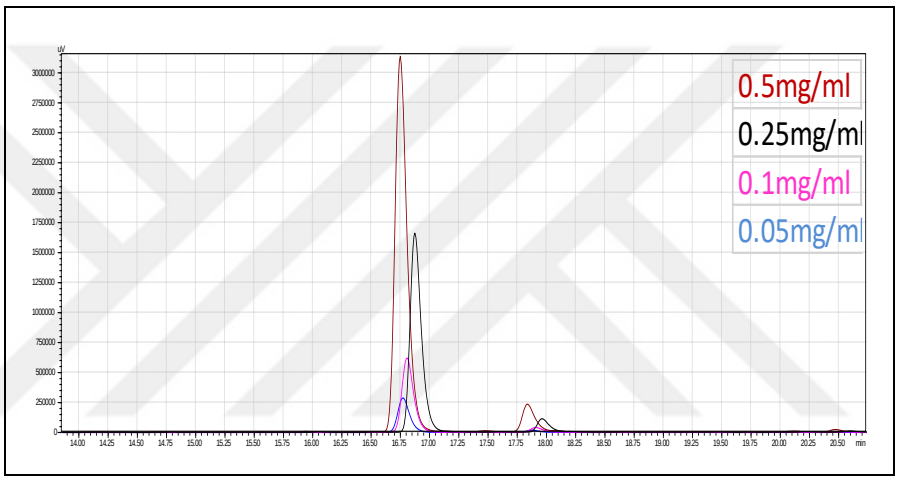


Figure 5.22 HPLC chromatogram of standard t-resveratrol for *V. labrusca* cv. Isabel (Second study)

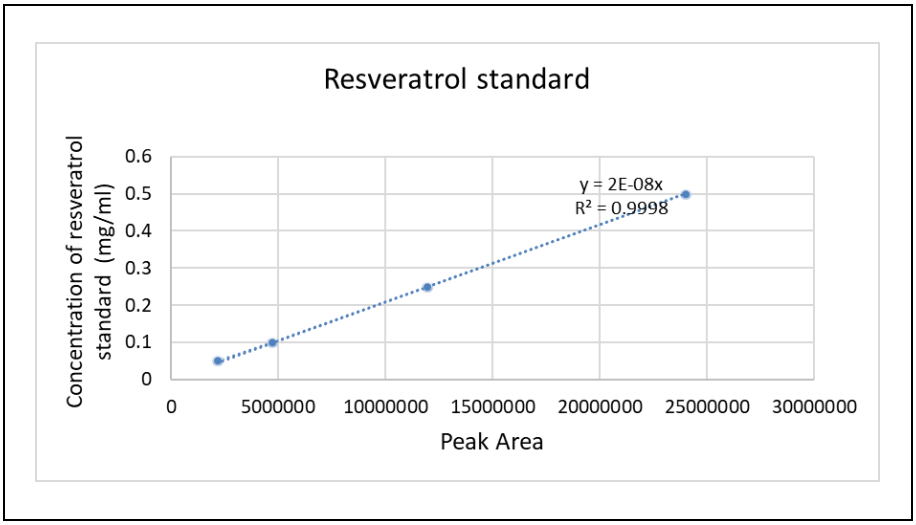


Figure 5.23. The calibration curve of standard t-resveratrol *V. labrusca* cv. Isabel (Second study)

In control samples of *V. labrusca* cv. Isabel CSC, the extracellular t-resveratrol was found on day 1 (7.768 mg/L) and day 3 (3.995 mg/L). After day 3, no extracellular t-resveratrol was found in control samples of *V. labrusca* cv. Isabel CSC (Figure 5.24 a). Also, intracellular t-resveratrol was found on day 1 (3.70 mg/L) and day 3 (3.66 mg/L). After day 3, no intracellular t-resveratrol was found in control samples of *V. labrusca* cv. Isabel CSC (Figure 5.24 b). In the *V. labrusca* cv. Isabel cell culture treated with MeJA (100 µM), extracellular t-resveratrol significantly increased after day 5 (when cells were treated with MeJA). On the 7th day, the level of extracellular t- resveratrol reached around 28,23 mg/L and after day 7 it decreased up to 4.31 mg/L (day 13) (Figure 5.24 a). As shown in Figure 5.24 b, intracellular t-resveratrol was also affected by adding MeJA on day 5. On the 11th day, the level of intracellular t- resveratrol reached around 6.78 mg/L, and no intracellular t-resveratrol was found on day 13. Resveratrol stimulated its production and it stopped the growth of cells because cell division metabolism is antagonistic to its production (258). Although the inhibition of cell growth was an important phenomenon, t-resveratrol production was not affected by cell decrease since high levels of resveratrol were produced after MeJA was added. Figure 5.25 represent an HPLC chromatogram showing the peaks of extracellular and intracellular t-resveratrol production in *V. labrusca* cv. Isabel.

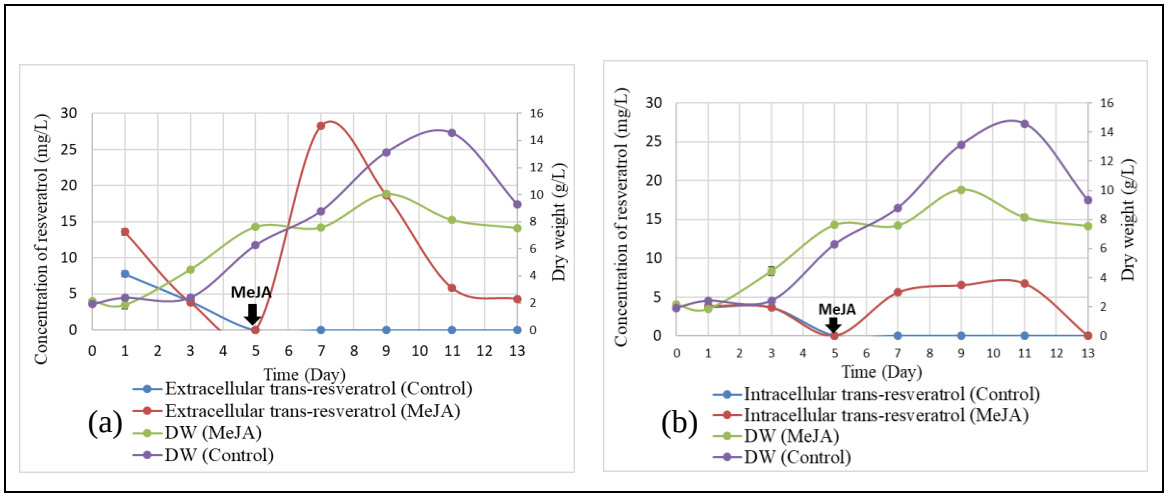


Figure 5.24 t-resveratrol production in *Vitis labrusca* cv. Isabel CSC. (a) Extracellular t-resveratrol, and (b) Intracellular t-resveratrol

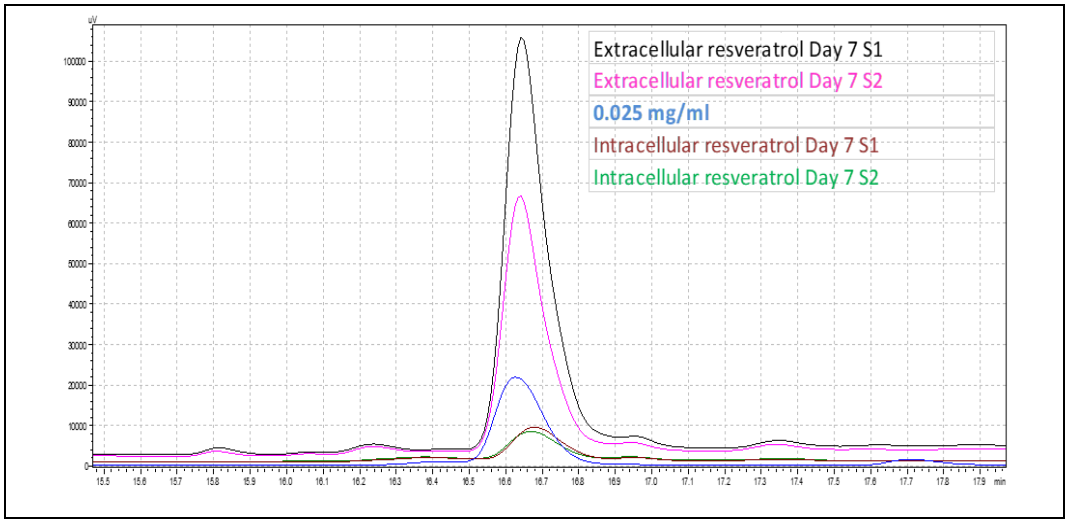


Figure 5.25. HPLC chromatograms of extracellular and intracellular t-resveratrol from *V. labrusca* cv. Isabel CSC (Day 7 (duplicate) after elicitation with MeJA)

5.9. SUGAR ANALYSIS IN *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE

First study

For *V. labrusca* cv. Isabel CSC, sucrose was decomposed by the cell wall into fructose and glucose after 24 hours. Sucrose concentration at day 0 was around 30 g/L and decreased up to 0.45 g/L on day 13. Glucose was favorably consumed over fructose on *V. labrusca* cv. Isabel and cells entered their stationary phase when glucose was depleted. During sucrose hydrolysis, the growth of cells was slower compared to when it was completed and only glucose and fructose were present in the media. (Figure 5.26 a). Fructose and glucose were consumed at different specific rates (0.34/day and 0.18/day respectively) (Figure 5.26 b).
The sucrose concentration in elicited media of *V. labrusca* cv. Isabel was decomposed by the cell wall into fructose and glucose after 24 hours. (Figure 5.26 c). Sucrose concentration at day 0 was around 30 g/L and was decreased up to 0.48 g/L at day 9, and after day 9, no sucrose was found in elicited media of *V. labrusca* cv. Isabel cells. Fructose and glucose were consumed at maximum specific rates (0.32/day and 0.15/day respectively) (Figure 5.26 d). Based on these observations, the growth cells and resveratrol production were affected by decreasing glucose and fructose after day 3.

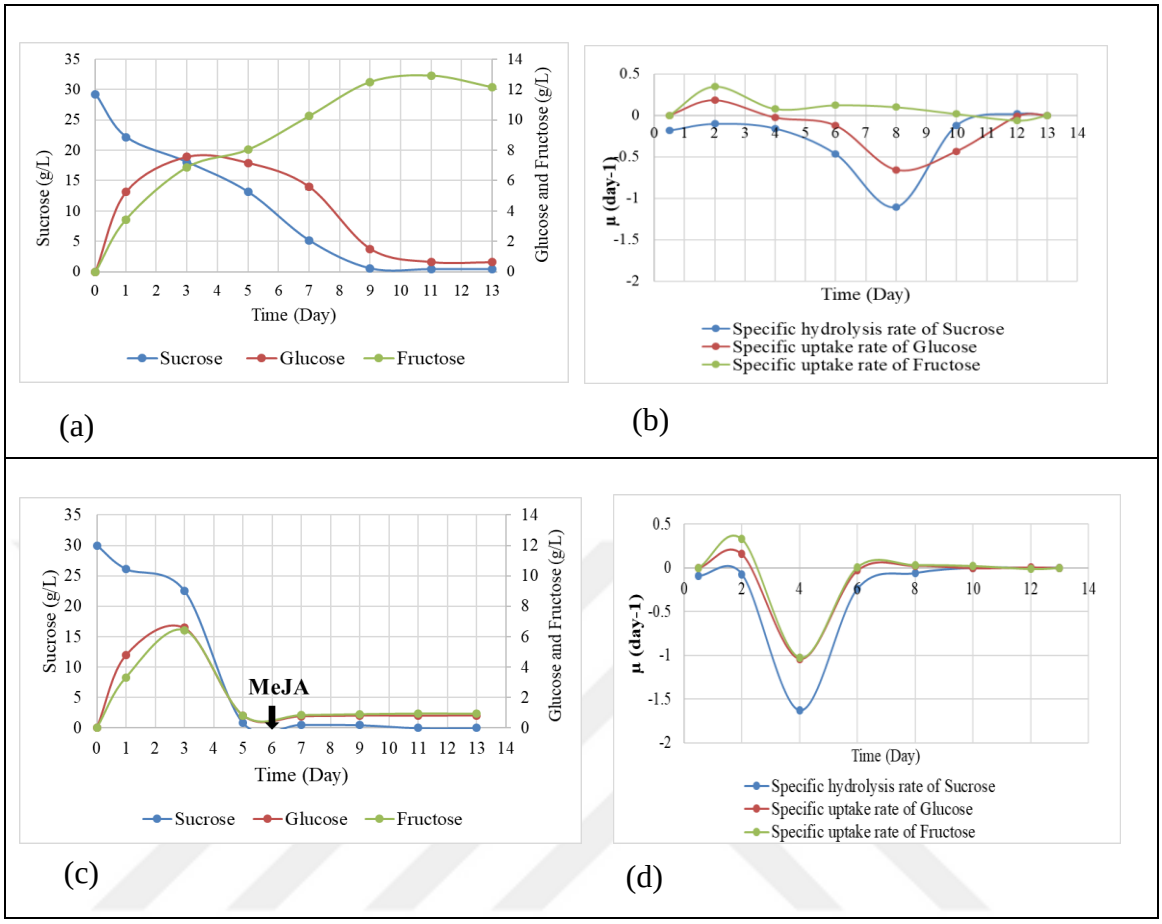


Figure 5.26. Sugar analysis in *V. labrusca* cv. Isabel CSC. (a) Control media, (b) Specific rate of sugar uptake-Control media, (c) Elicited media, and (d) Specific rate of sugar uptake-Elicited media (First study)

Second study

For *V. labrusca* cv. Isabel CSC, sucrose was decomposed by the cell wall into fructose and glucose after 24 hours. Sucrose concentration at day 0 was around 30 g/L and was decreased up to 0.02 g/L on day 29 and day 31 no sucrose concentration was found (Figure 5.27 a). Fructose and glucose were consumed at different specific rates. The maximum specific rate was 0.28/day and 0.37/day for fructose and glucose, respectively. (Figure 5.27 b). Glucose was favorably consumed over fructose on *V. labrusca* cv. Isabel and cells entered their stationary phase when glucose was consumed. During sucrose hydrolysis, the growth of cells was slower compared to when it was completed and only glucose and fructose were present in the media.

The sucrose concentration in elicited media of *V. labrusca* cv. Isabel cells was decomposed by the cell wall into fructose and glucose after 24 hours. Sucrose concentration at day 0 was around 30 g/L and was decreased up to 0.57 g/L at day 11, and after day 11, no sucrose was found in elicited media of *V. labrusca* cv. Isabel cells (Figure 5.27 c). Fructose and glucose were consumed at maximum specific rates (0.29/day and 0.17/day respectively) (Figure 5.27 d).

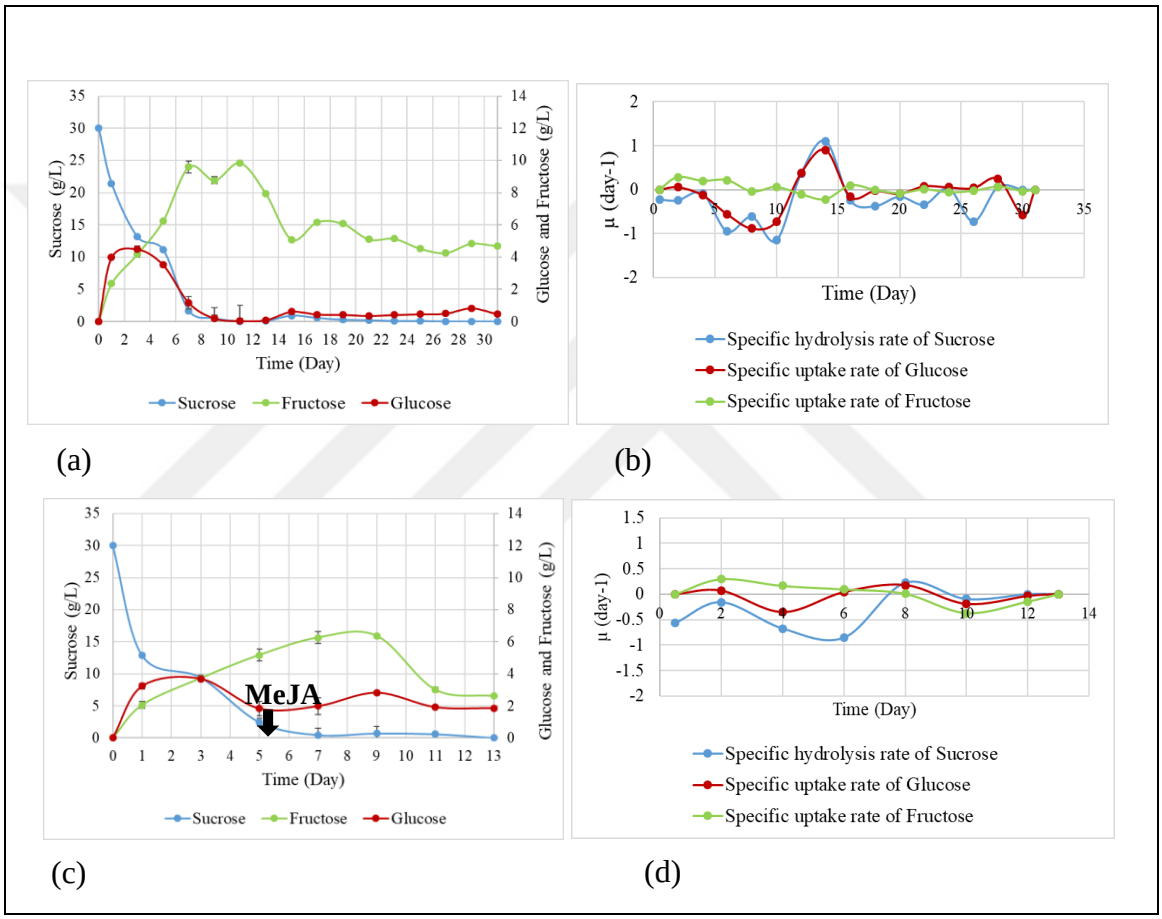


Figure 5.27 Sugar analysis in *V. labrusca* cv. Isabel CSC. (a) Control media, (b) Specific rate of sugar uptake- Control media, (c) Elicited media, (d) Specific rate of sugar uptake- Elicited media (Second study)

Third study

For *V. labrusca* cv. Isabel CSC, cultivated into VlSCIM3, was decomposed by the cell wall into fructose and glucose after 24 hours. Sucrose concentration at day 0 was around 30 g/L and was decreased up to 0.2 g/L at day 17 (Figure 5. 28 a). Fructose and glucose were consumed at different specific rates (0.44/day and 0.73/day respectively) (Figure 5.28 b).

Glucose was favorably consumed over fructose on *V. labrusca* cv. Isabel and cells entered their stationary phase when glucose was decreased. During sucrose hydrolysis, the growth of cells was slower compared to when it was completed and only glucose and fructose were present in the media.

Figures 5.26a, 5.27a and 5.28a, and 5.54 indicate that glucose seems to be preferentially taken up by the cells because the level of this hexose in the medium was lower than that of fructose when they were hydrolyzed. These findings suggest the accumulation of fructose in the culture medium of *V. labrusca* cv. Isabel is not linked to an inability to utilize this hexose, but rather to the fact that glucose is a better substrate for cell respiration, and has been attributed to a higher affinity of the sugar transport proteins for the glucose when compared to fructose as reported for CSC of *Catharanthus roseus* (273), carrot (274) (275), soybean (276) and bean (277).

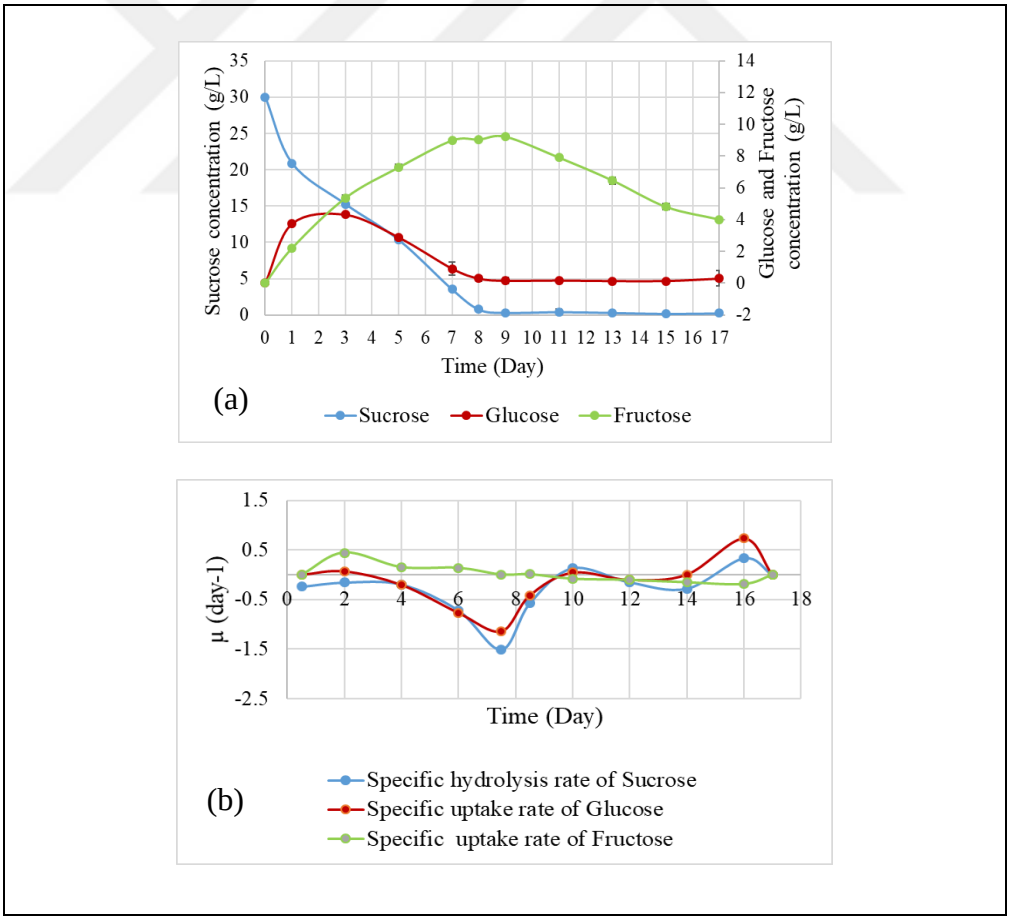


Figure 5.28. Sugar analysis in *V. labrusca* cv. Isabel CSC without casein hydrolysate into media. (a) Sugar graphs, and (b) Specific rate of sugar uptake

5.10. PROCESS SCALE-UP OF *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE IN STR

5.10.1. Biomass analysis in *V. labrusca* cv. Isabel cell suspension culture cultivated in STR

The results performed in flasks prompted tests to scale- production in a 6 L bioreactor (using 3 L volume). We have focused on the scale-up of *V. labrusca* cv. Isabel CSC by optimizing parameters as shown in tables 4.6 - 4.8.

In the first trial, the airflow was set at 0.02 vvm (3x0.02vvm=0.06L/min) based on the article (Donnez et al. 2011; Jeandet et al. 2014) (136) (193). The inoculum cell density was set at 20 g/L. Cells showed a quick change of color from clear to yellow brown within 24 hours of batch and on day 5 the cells showed dark brown color which is a result of phenolic compound production and death of cells due to stress factors. Their growth was inhibited showing only 68,43 g of fresh weight on day 5 of the batch (Figure 5.53 a). Figure 5.29 shows the changes in this batch, whereas Figure 5.54n shows the sugar consumption graph. Thus, based on the sugar consumption graph, cell death was not dependent on the media composition and lack of carbon source, but on the stress factors caused by the level of oxygen correlated to low initial cell density.

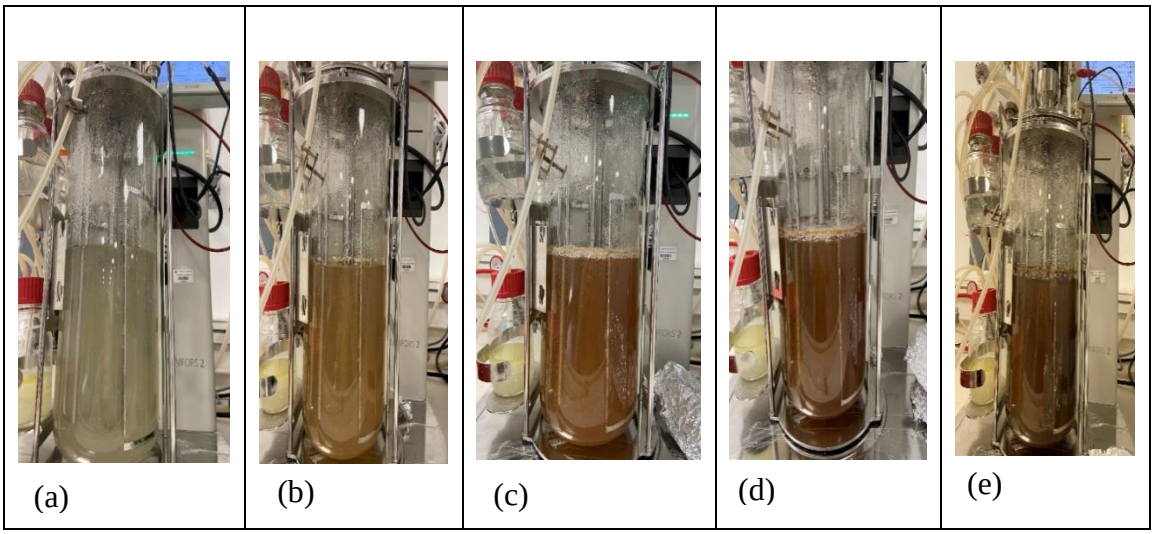


Figure 5.29. *V. labrusca* cv. Isabel CSC in the bioreactor (trial 1) at days (a) 0, (b) 1, (c) 2, (d) 3, and (e) 5

In the second trial, the airflow was set at 0.15 vvm (3Lx0.15vvm=0.45 L/min) based on the article (Lambert et al, 2019) (260). The cells showed a change of color from clear to yellow-brown on the second day of the batch. The foaming problem increased and cells were mostly dead on day 3 as can be seen in Figure 5.30. The final cell mass was decreased to 29.71 g (Figure 5.53a). Figure 5.30 shows the changes in this batch, whereas Figure 5.54m shows the sugar consumption graph. Thus, based on the sugar consumption graph, cell death was not dependent on the media composition and lack of carbon source, but on the stress factors caused by the level of oxygen correlated to low initial cell density, and foaming formation.

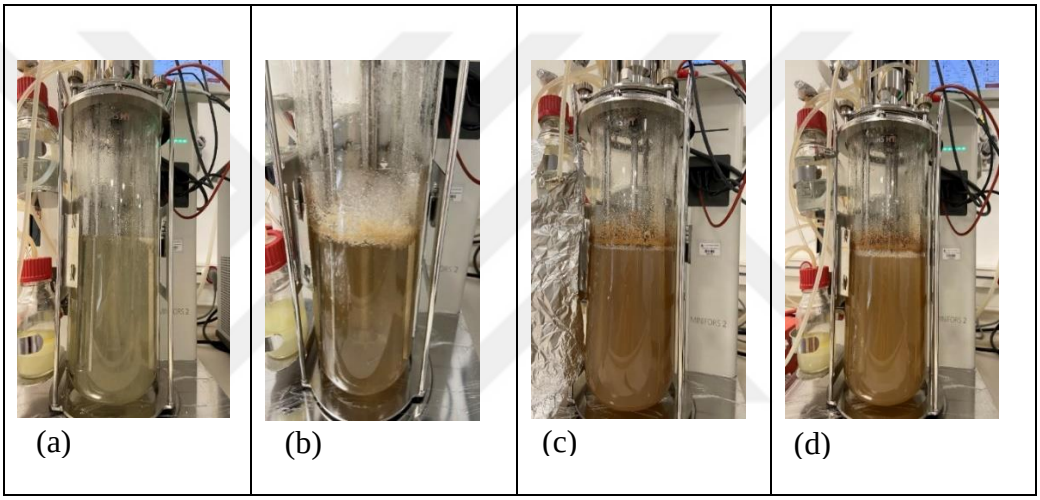


Figure 5.30. *V. labrusca* cv. Isabel CSC in the bioreactor trial 2 at days (a) 0, (b) 1, (c) 2, and (d) 3

In the third trial, the airflow was set at 0.08 vvm (0.08 vvmx3L=0.24 L/min) as the average found between the airflow set in the first trial and the airflow set in the second trial. The inoculum cell density was set to 20 g/L. Cells showed a quick change of color from clear to yellow brown within 24 hours of batch and on day 5 the cells showed dark brown color which is a result of phenolic compound production and death of cells due to stress factors. The foaming problem increased in this trial also. Their growth was inhibited showing only 69,85 g of fresh weight on day 5 of the batch (Figure 5.53a). Figure 5.31 shows the changes in this batch, whereas Figure 5.54l shows the sugar consumption graph. Thus, based on the sugar consumption graph, cell death was not dependent on the media composition and lack of carbon source, but on the stress factors caused by the level of oxygen correlated to low initial cell density and foaming formation.

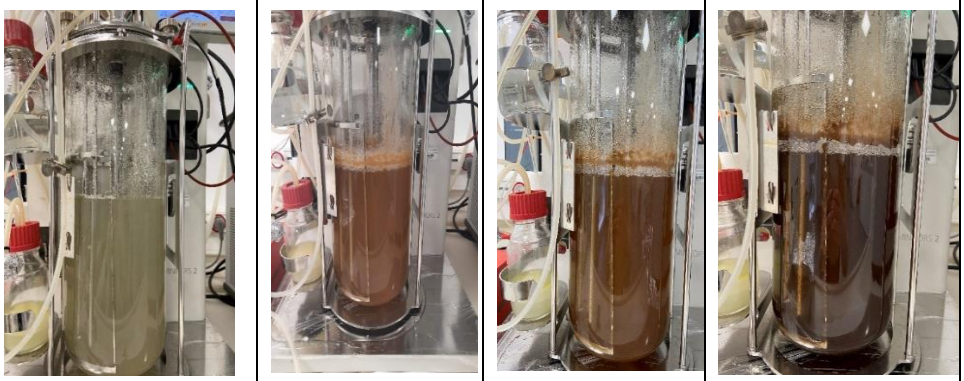


Figure 5.31. *V. labrusca* cv. Isabel CSC in the STR (trial 3) at days (a) 0, (b) 1, (c) 2, and (d) 5.

In the fourth trial, the initial airflow was set at 0.02 vvm (0.02vvmx3L=0.06 L/min) based on an article (136) (193). The inoculum cell density was set to 45 g/L. Cells showed a slow change of color from clear to yellow brown until day 5, then cells showed dark brown color which is a result of phenolic compound production and death of cells due to stress factors. Increasing the inoculum cell density, decreasing the initial airflow to 0.02 vvm, and using a PID control circuit between 0.02-0.08 vvm (0.06-0.24 L/min) to avoid the dissolved oxygen concentration drops below a certain threshold resulted in the successful growth of cells. The final fresh weight of the cells on day 5 of the batch was 172,06 g (Figure 5.53 b). Figure 5.32 shows the changes in this batch, whereas Figure 5.54k shows the sugar consumption graph.

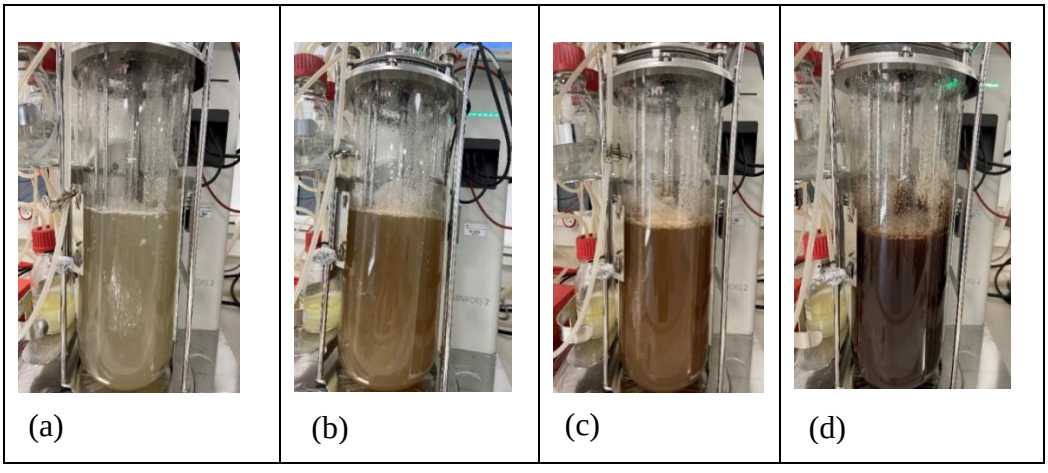


Figure 5.32. *V. labrusca* cv. Isabel CSC in the STR (trial 4) at days (a) 0, (b) 1, (c) 2, and (d) 5.

In trials 5 and 7, the initial airflow was set at 0.02 vvm (0.06 L/min) based on an article (136) (193), and the cells were cultivated with the presence of light as can be seen in Figures 5.33 and 5.34. The inoculum cell density was set to 45 g/L. Cells showed a clear color (white) until day 3, then cells started to show a slow change from clear to yellow brown until day 5 where cells showed dark brown color which is a result of phenolic compound production and death of cells due to stress factors. Increasing the inoculum cell density, decreasing the initial airflow to 0.02 vvm, using a PID control circuit between 0.02-0.15 vvm (0.06-0.45 L/min) to avoid the dropping of dissolved oxygen concentration below a certain threshold, and using light resulted in the successful growth of cells. The difference between trials 5 and 7 was the batch time and the day of the airflow changes from 0.24 L/min to 0.45 L/min in a PID control circuit. In trial 5, batch time was 5 days and the final fresh weight of cells was 274.07 g (Figure 5.53b), whereas in trial 7, batch time was 6 days and the final fresh weight of cells was 301.13 g (Figure 5.53 c). Figures 5.33 and 5.34 shows the changes in this batch, whereas Figures 5.54i and 5.54j shows the sugar consumption graphs for trial 5 and trial 7, respectivelly.

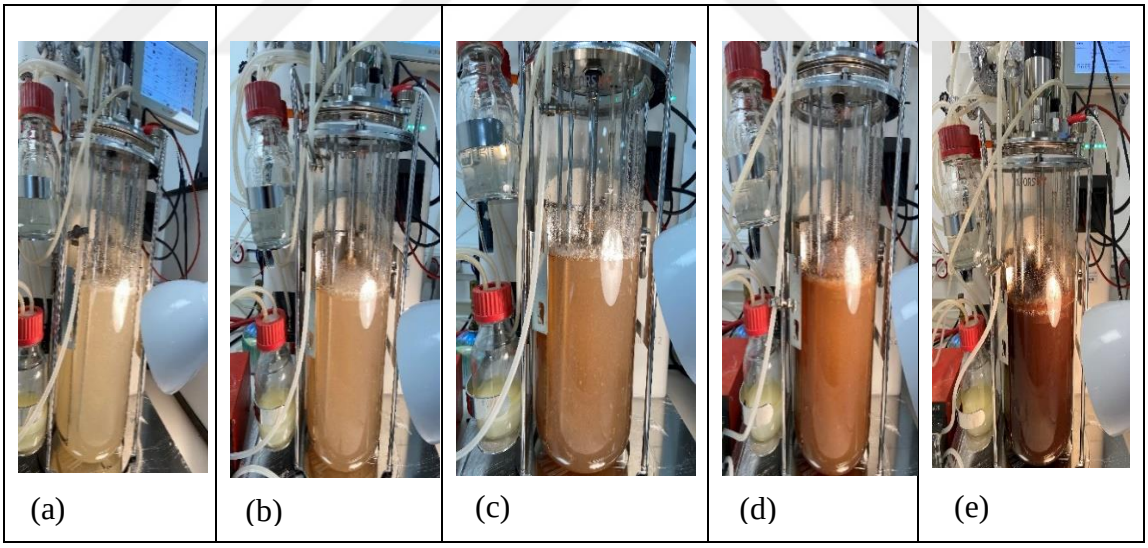


Figure 5.33. *V. labrusca* cv. Isabel CSC in the STR (trial 5) at days (a) 0, (b) 1, (c) 2, (d) 3, and (e) 5

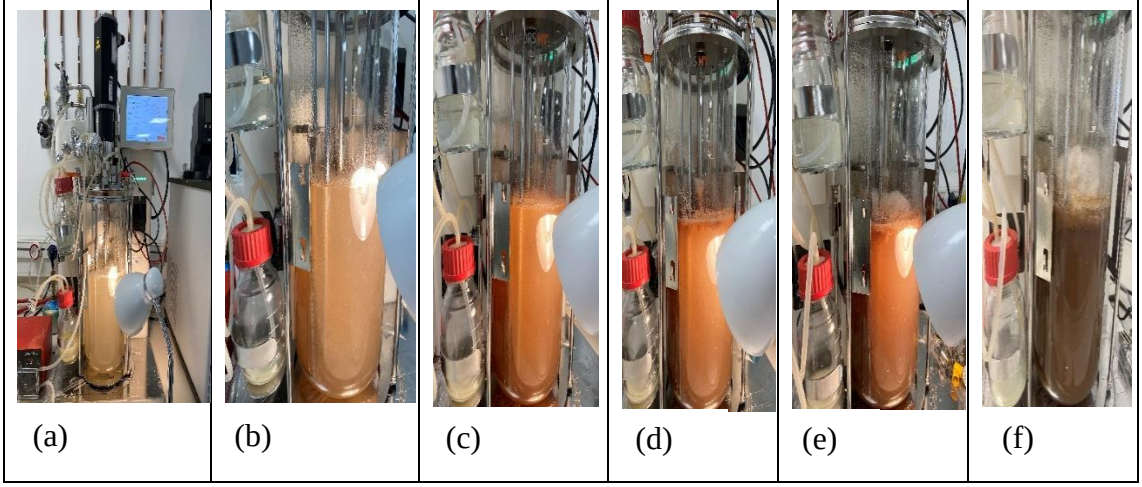


Figure 5.34. *V. labrusca* cv. Isabel CSC in the STR (trial 7) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 5, and (f) 6

From trial 10 to trial 14, and in trial 17, all parameters were performed the same; oxygen values were between 0.02-0.26 vvm (0.06-0.8 L/min) kept in a PID control circuit, 45 g/L was used as inoculum density, and keeping the cells in dark condition. These conditions did not give amelioration of cell growth. The highest amount of dry weight was obtained in trial 13 which was 11.8 g DW/3L (Figure 5.53 d). Cells were viable up to day 5 based on the photos assessed by fluorescence microscopy of cells treated with FDA (Figure 5.36, 5.38, 5.40, 5.42, 5.44, and 5.50). The DW on all trials in an STR is lower than the DW obtained in Erlenmeyer (cultivated in the same media composition and dark conditions on both Erlenmeyer and STR (Figure 5.53d). Figures 5.35, 5.37, 5.39, 5.41, 5.43, and 5.49 show the changes in those trials, whereas Figures 5.54 a, d, e, f, g, and h show the sugar consumption graphs for trials 10-14 and trial 17. *V. labrusca* cv. Isabel CSC cultivated into media composition without CH (trials 15 and 16) showed longevity for 8 days based on the photos assessed by fluorescence microscopy of cells treated with FDA in both trials (Figure 5.46 and 5.48). In trial 15, the dry weight achieved was 13.12 g/3L while in trial 16 was 7.22 g/3 L (Figure 5.53 e). Figures 5.45 and 5.47 show the changes in those trials, whereas Figures 5.54 b and c show the sugar consumption graphs for trials 16 and 15. In trial 18, cells were elicited with MeJA, which started to show a slow change from clear to yellow-brown until day 5 when cells showed dark red color (comparable color with the color of red wine) which is a result of phenolic compound production. Cell growth affected in MeJA-treated cells was

shown also by cell viability measured by fluorescence microscopy of cells treated with FDA. The dry weight achieved was 8 g DW/3L (Figures 5.51 and 5.52).

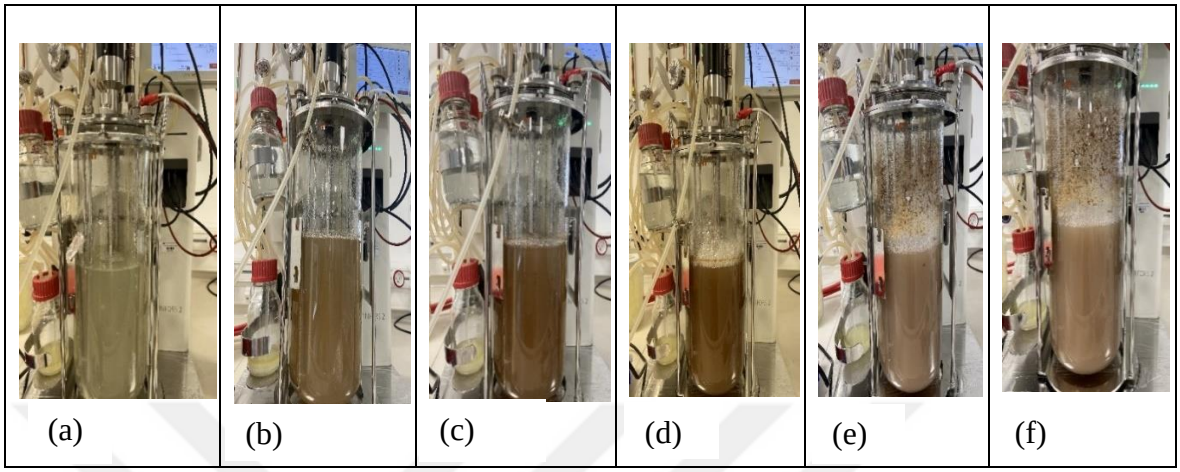


Figure 5.35. *V. labrusca* cv. Isabel CSC in the STR (trial 10) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

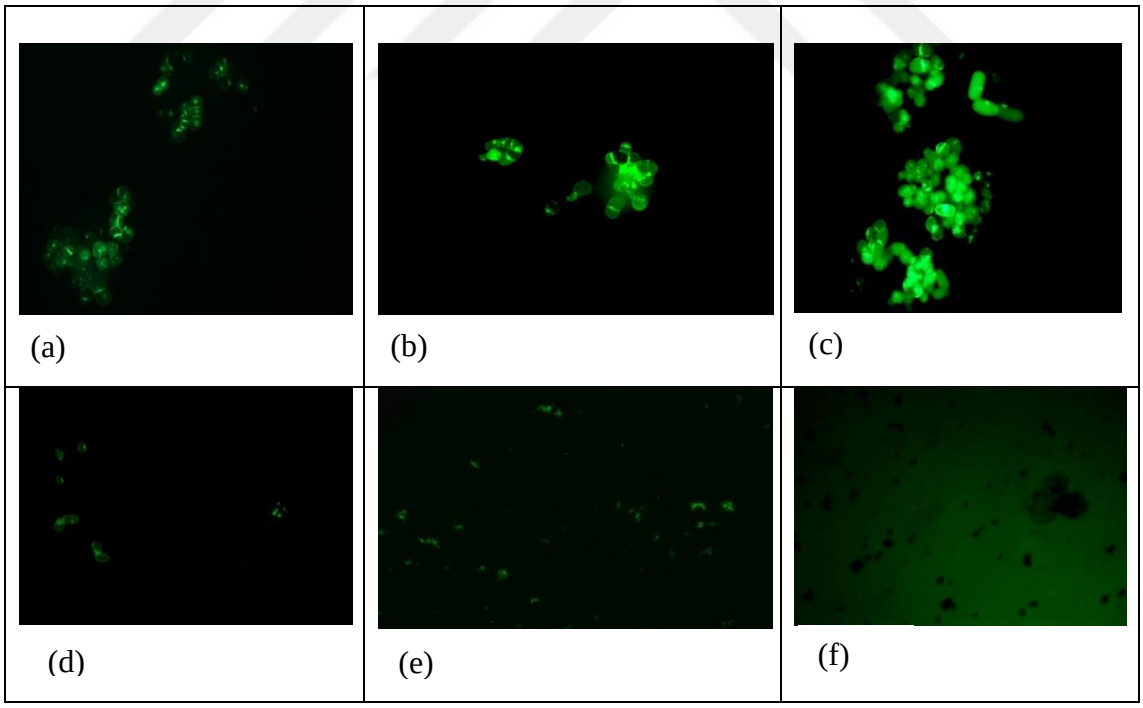


Figure 5.36. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 10) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

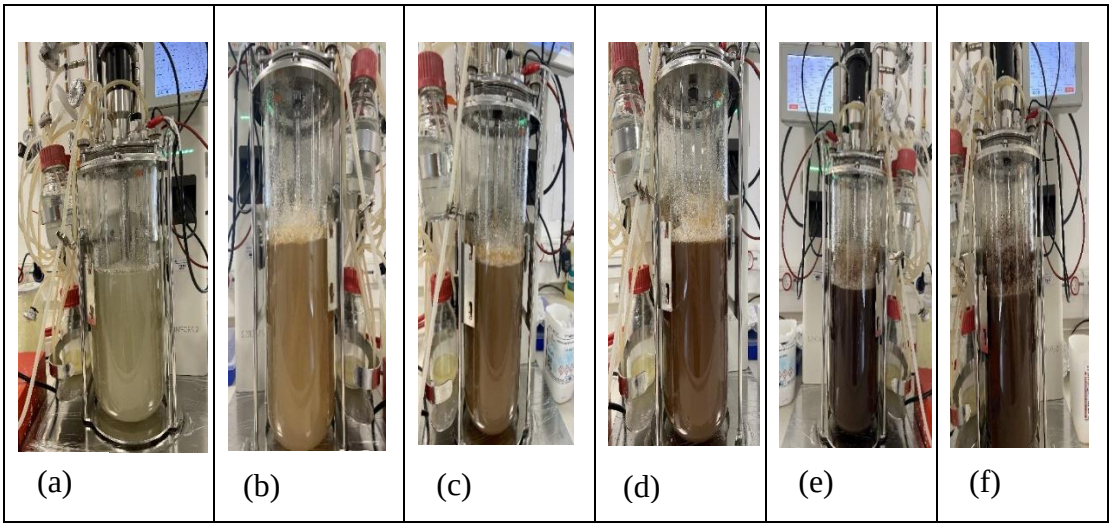


Figure 5.37. *V. labrusca* cv. Isabel CSC in the STR (trial 11) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

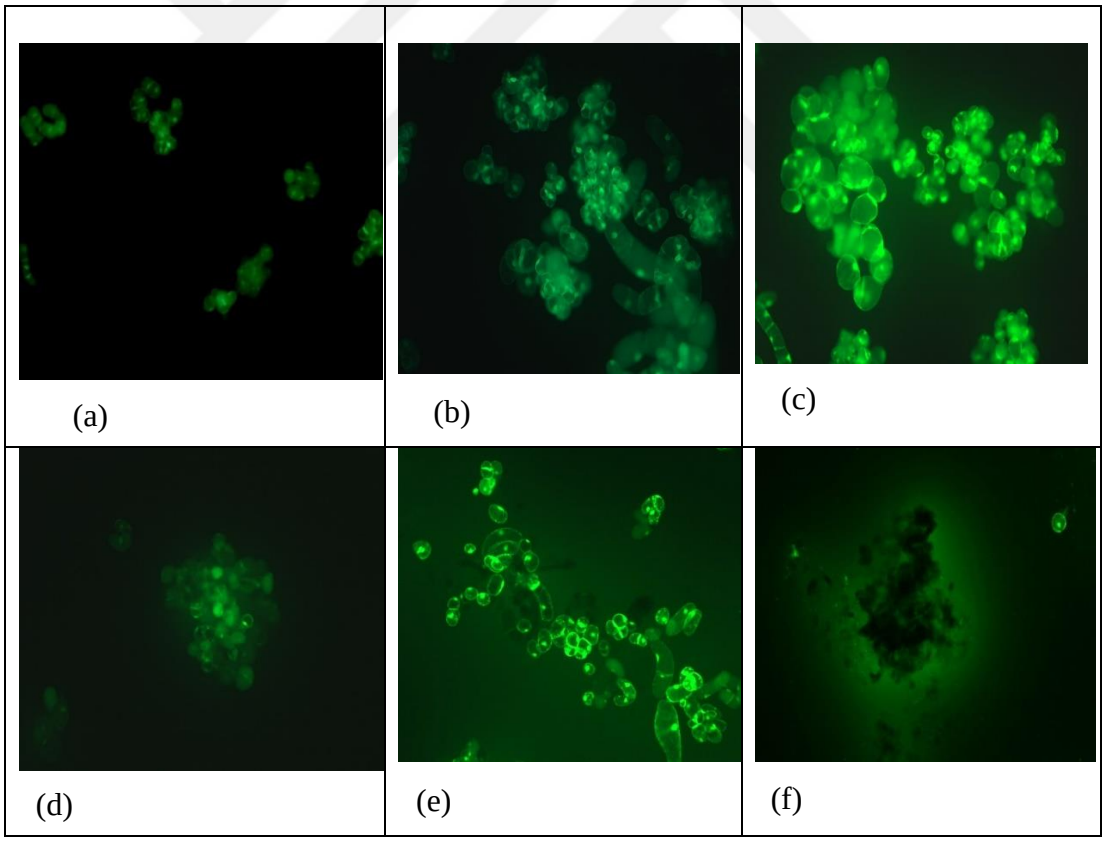


Figure 5.38. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 11) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

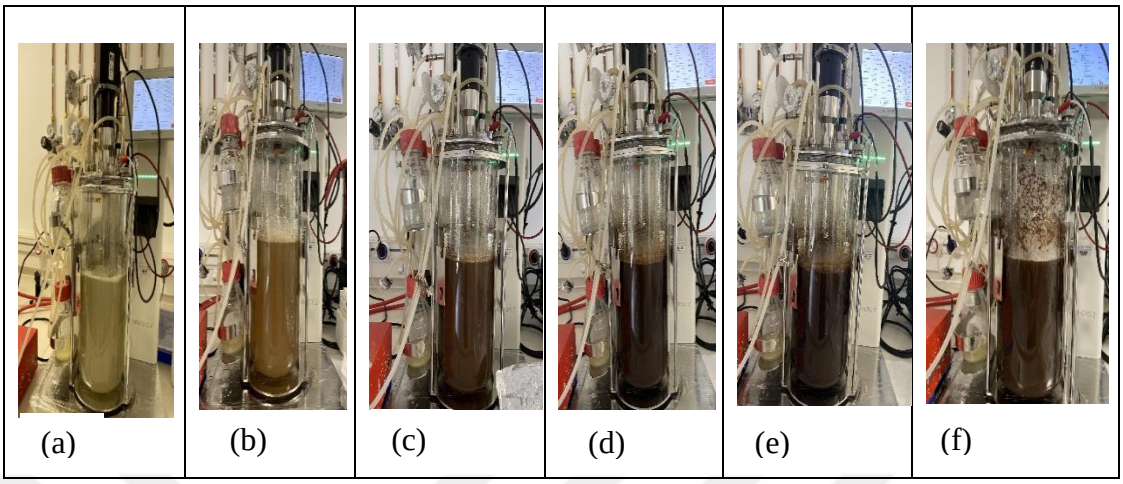


Figure 5.39. *V. labrusca* cv. Isabel CSC in the STR (trial 12) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

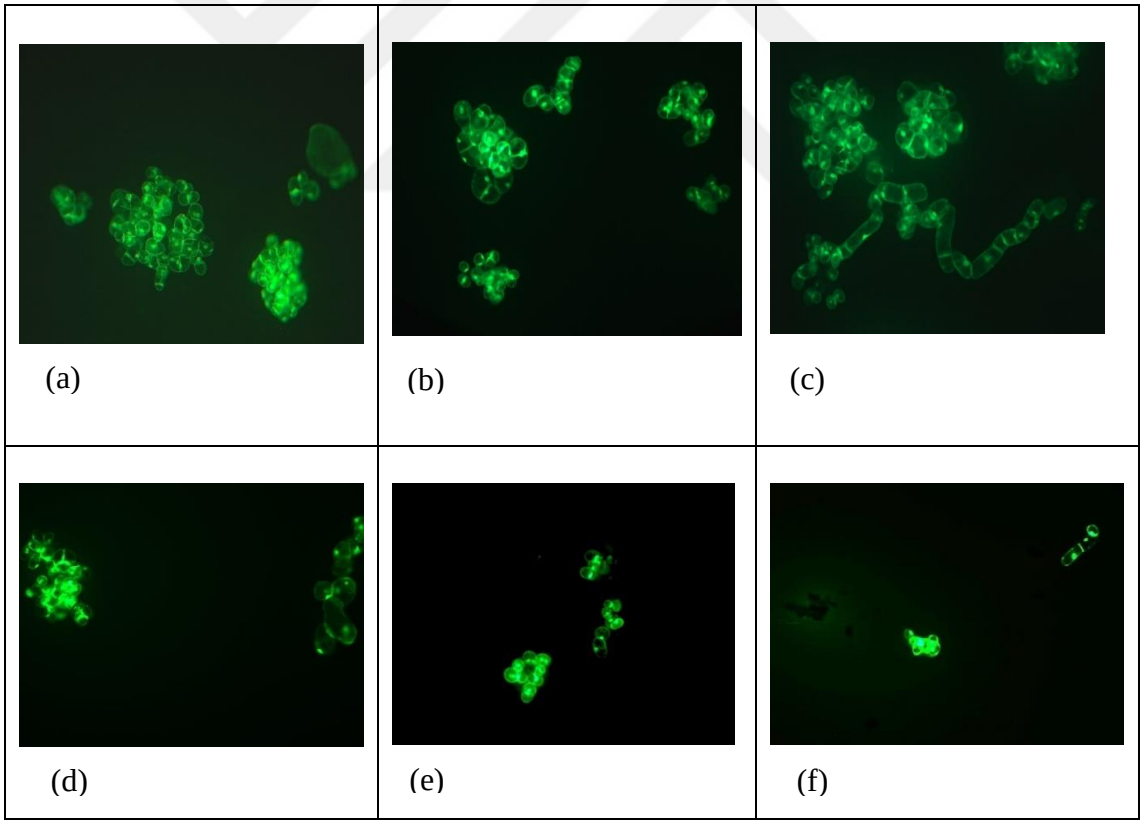


Figure 5.40. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 12) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

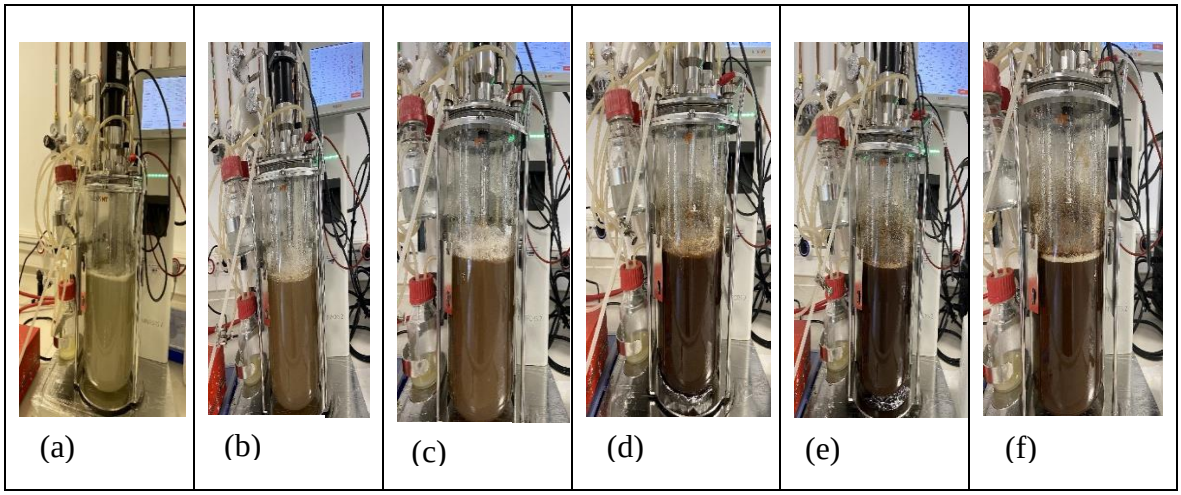


Figure 5.41. *V. labrusca* cv. Isabel CSC in the STR (trial 13) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

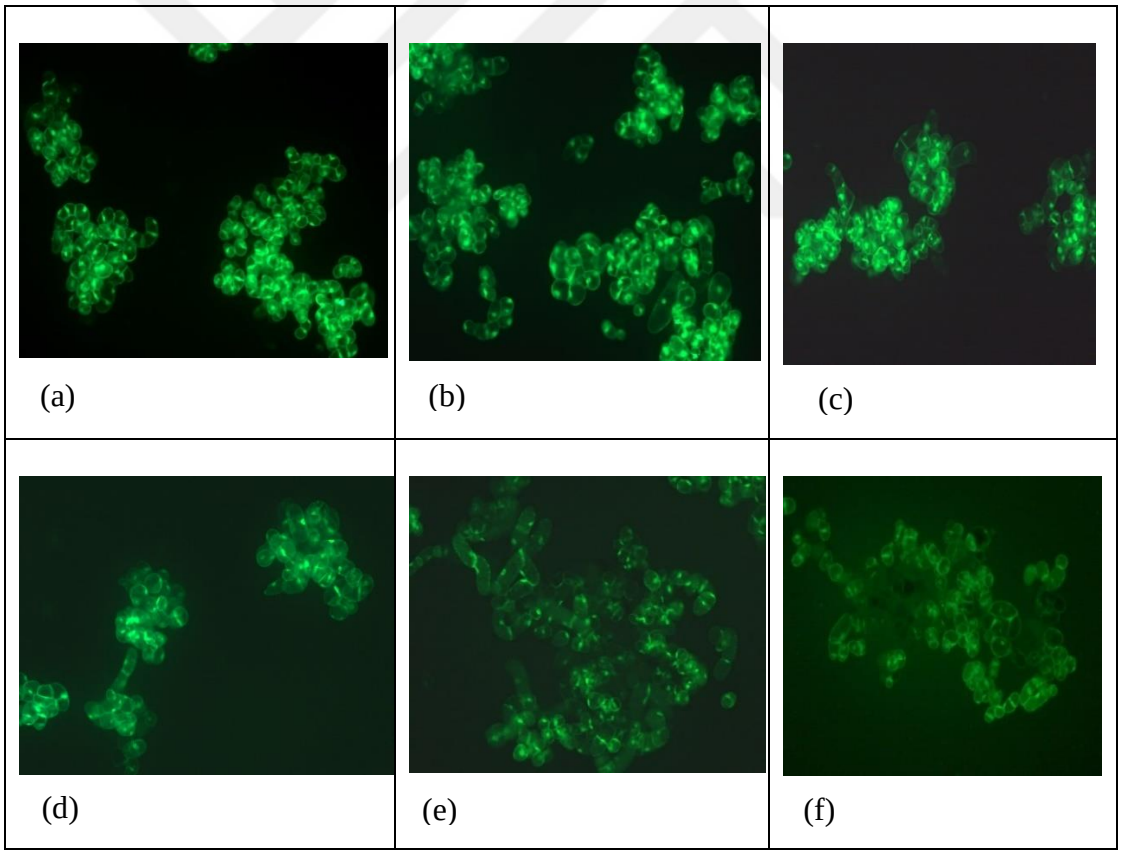


Figure 5.42. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 13) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

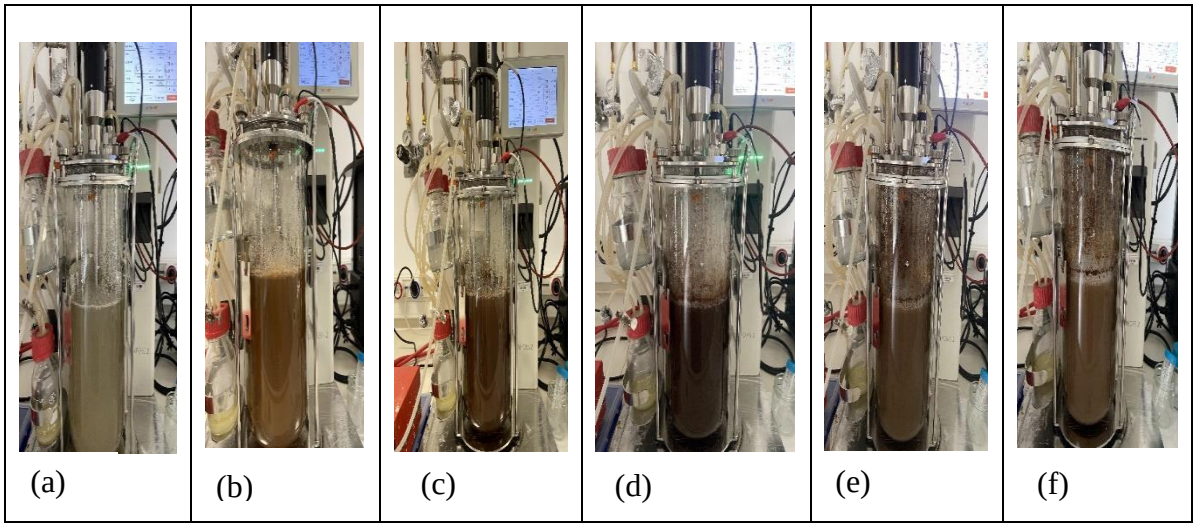


Figure 5.43. *V. labrusca* cv. Isabel CSC in the STR (trial 14) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

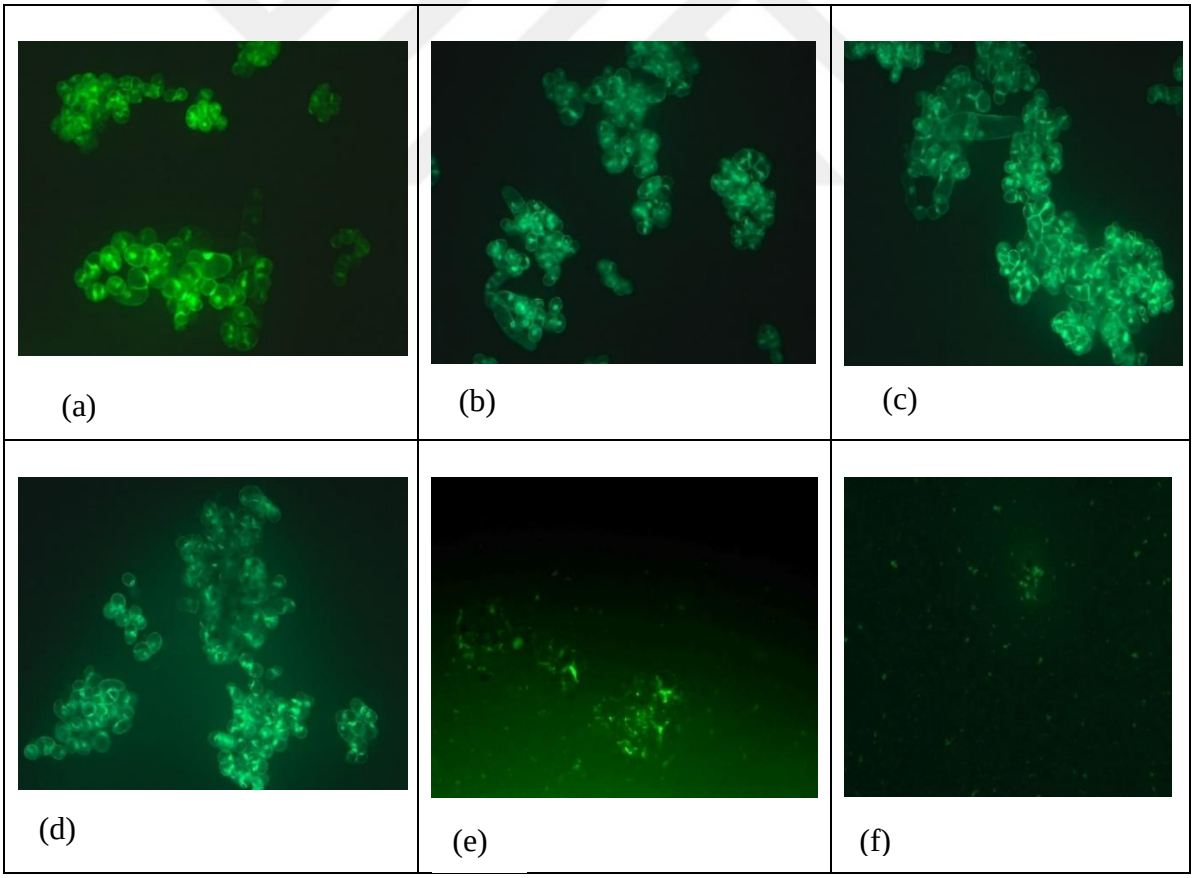


Figure 5.44. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 14) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5

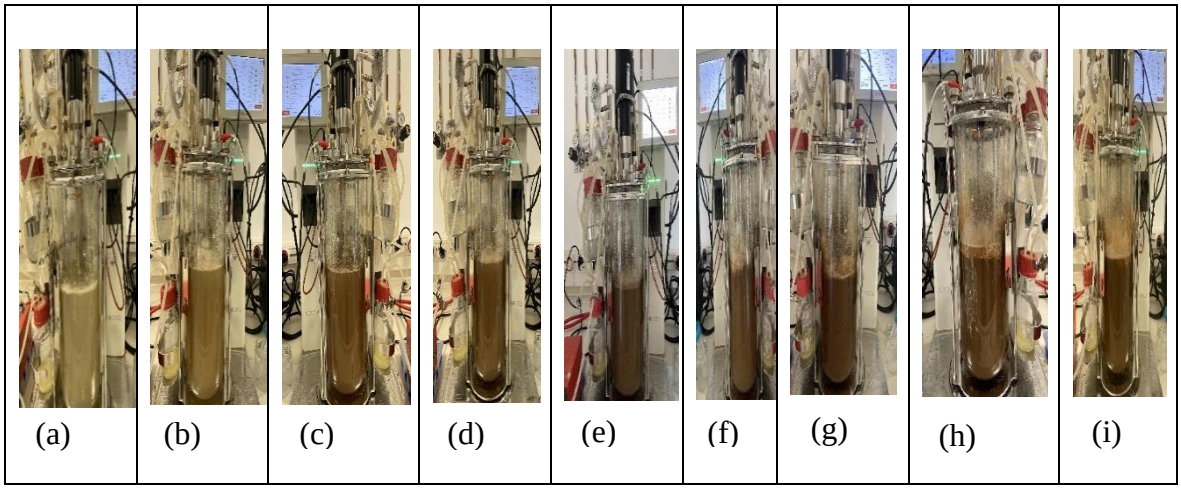


Figure 5.45. *V. labrusca* cv. Isabel CSC in the STR (trial 15) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, (f) 5, (g) 6, (h) 7, and (i) 8

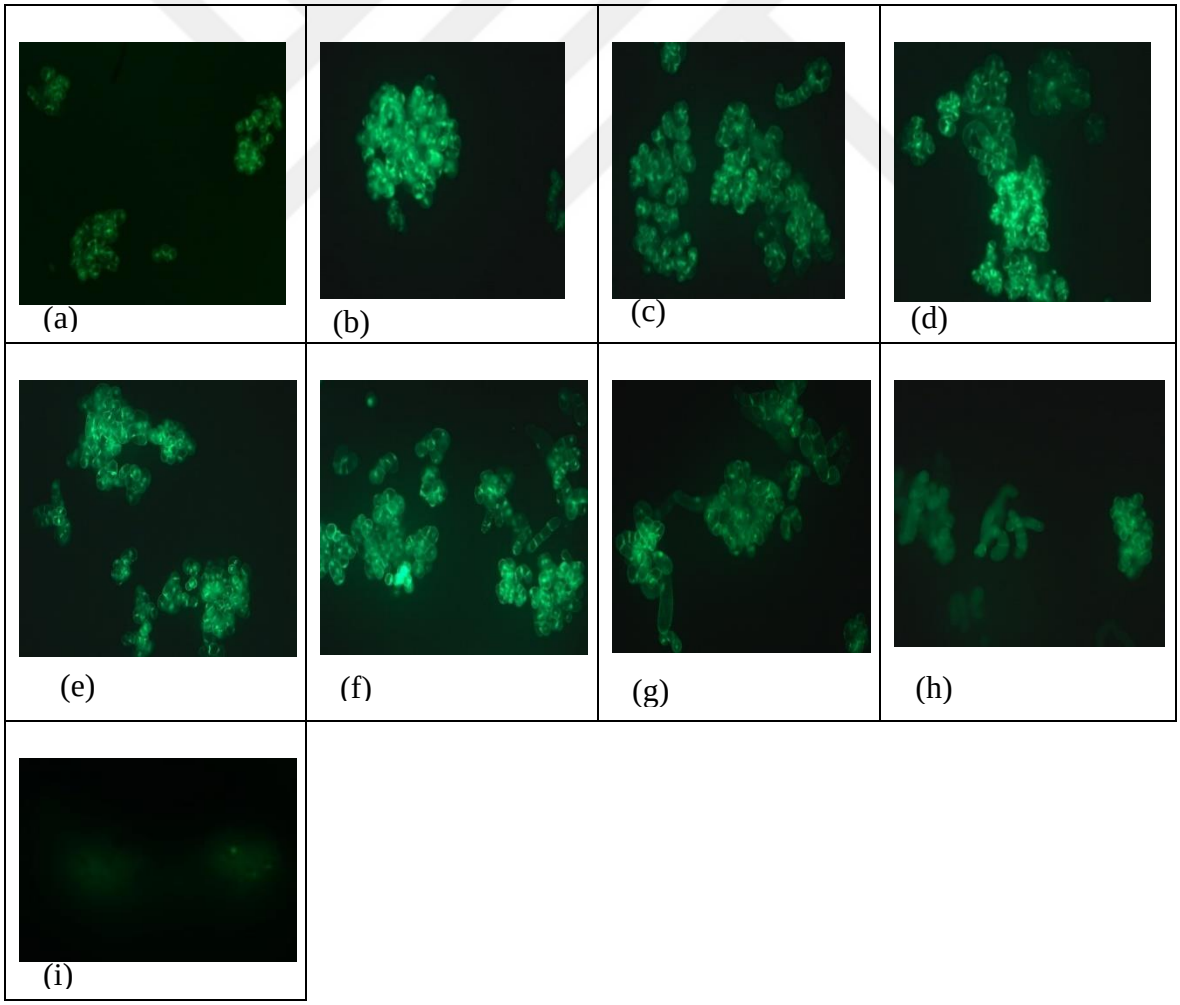


Figure 5.46. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 15) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, (f) 5, (g) 6, (h) 7, and (i) 8

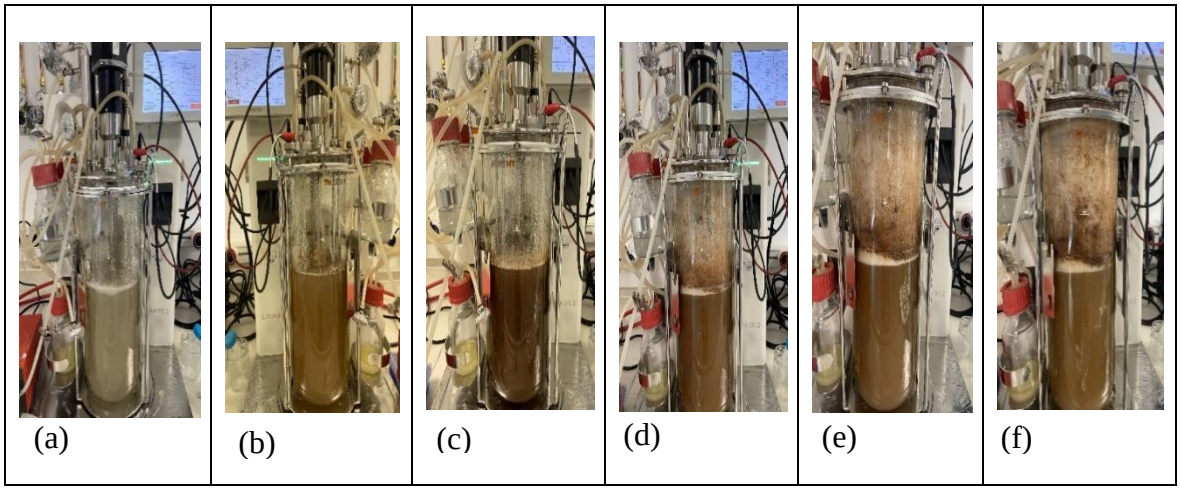


Figure 5.47. *V. labrusca* cv. Isabel CSC in the STR (trial 16) at days (a) 0, (b) 1, (c) 4, (d) 6, (e) 7, and (f) 8

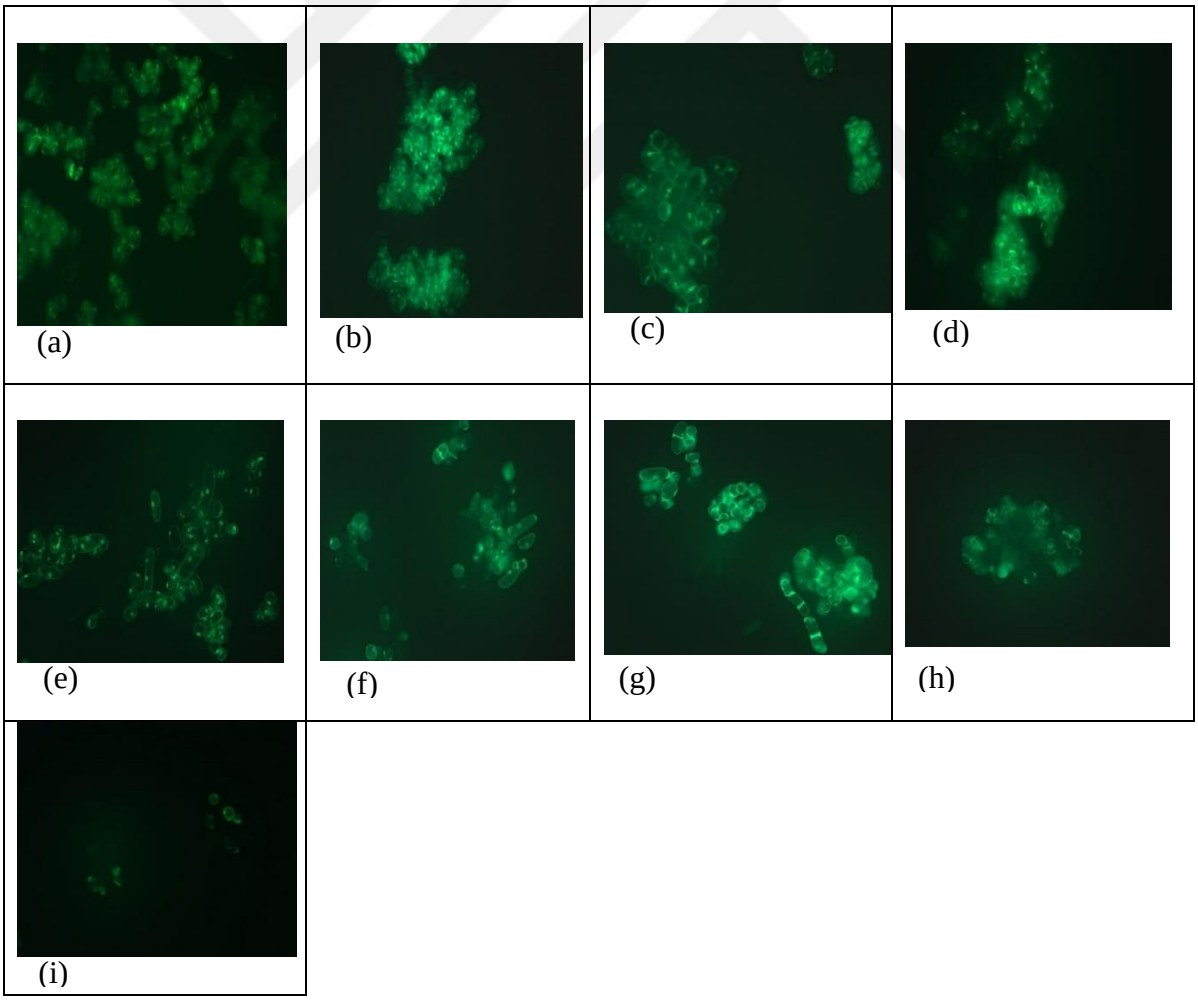


Figure 5.48. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 16) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, (f) 5, (g) 6, (h) 7, and (i) 8

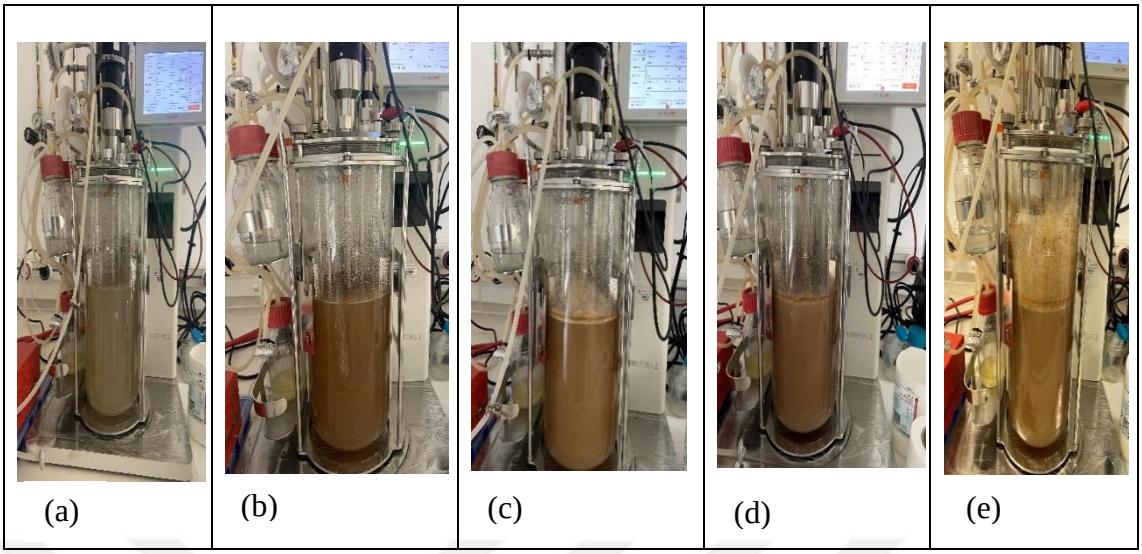


Figure 5.49. *V. labrusca* cv. Isabel CSC in the STR (trial 17) at days (a) 0, (b) 1, (c) 3, (d) 4, and (e) 5

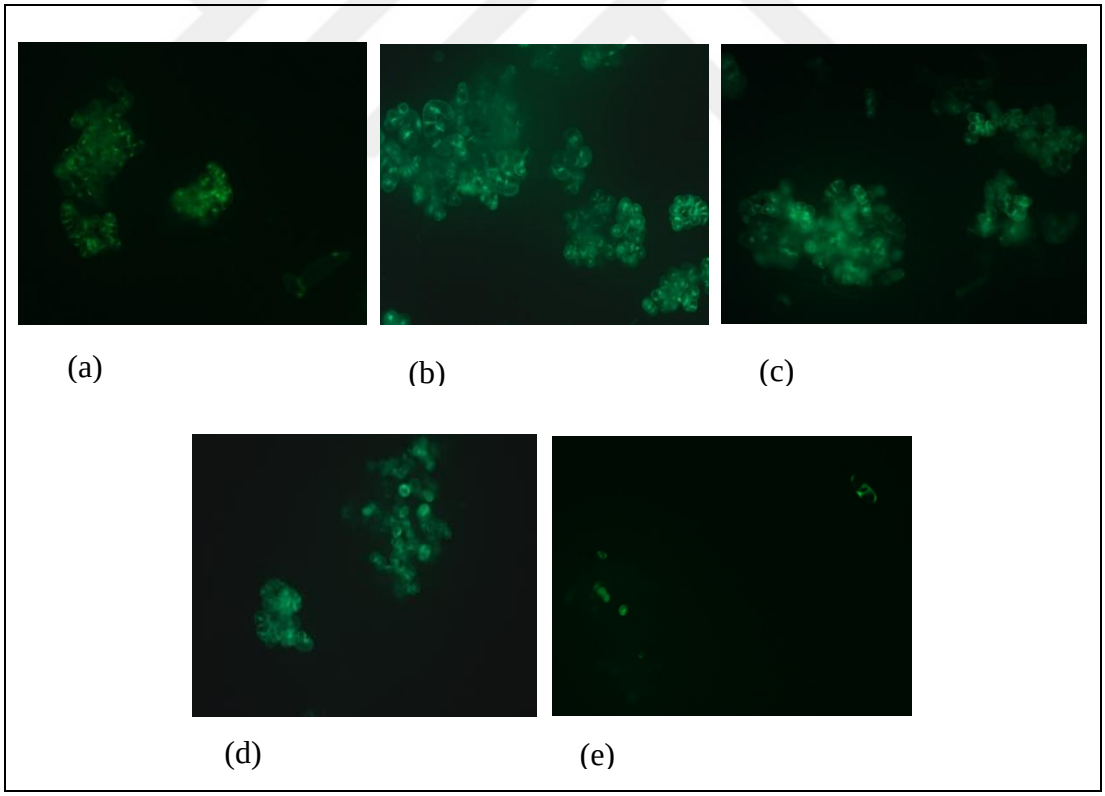


Figure 5.50. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 17) at days (a) 0, (b) 1, (c) 2, (d) 3, (e) 5

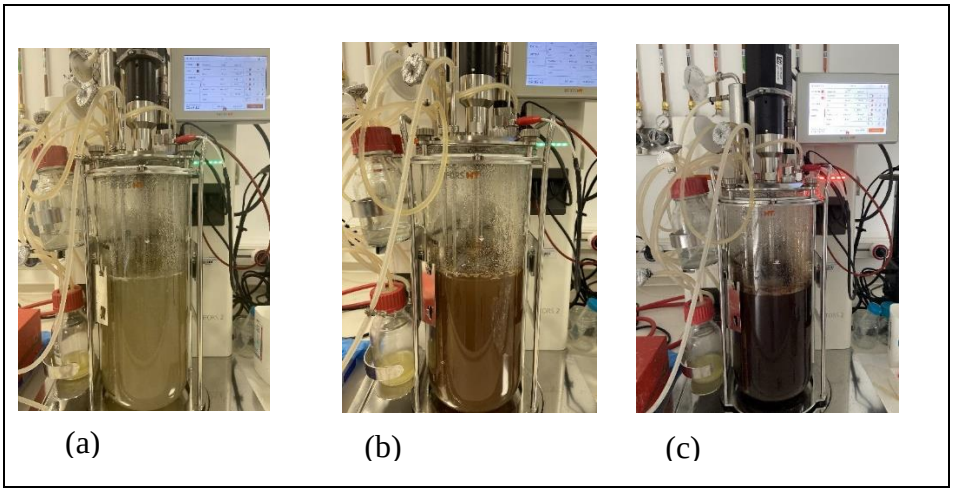


Figure 5.51. *V. labrusca* cv. Isabel CSC in the STR (trial 18) at days (a) 0, (b) 3, (c) 5

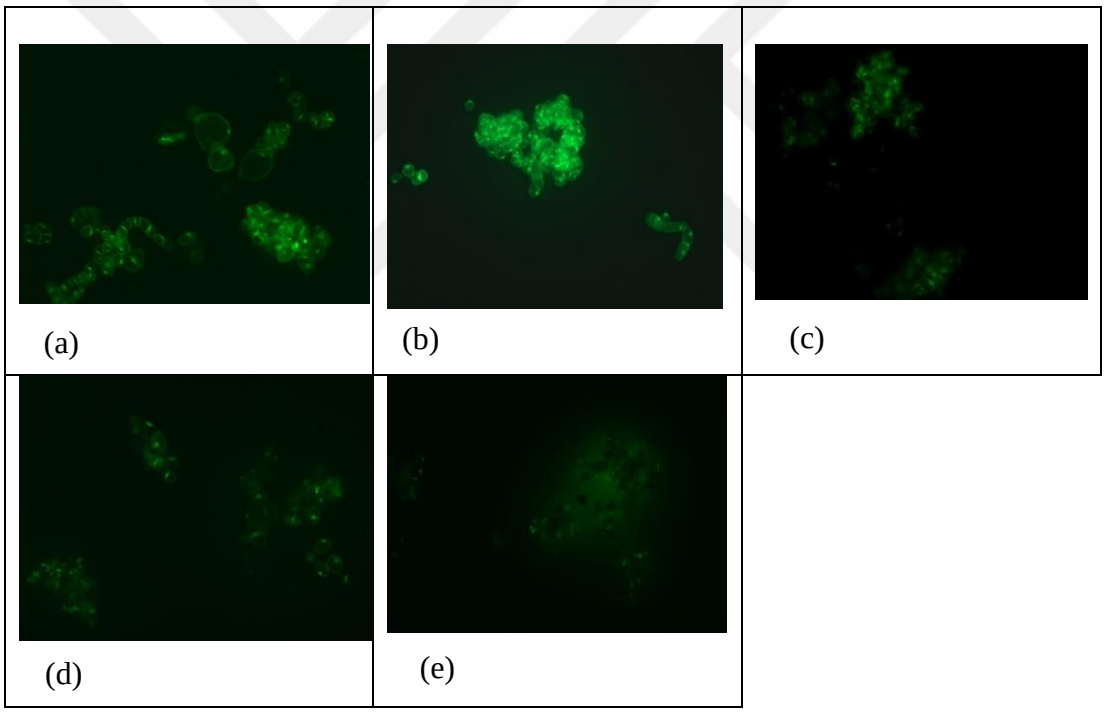


Figure 5.52. Cell viability of *V. labrusca* cv. Isabel CSC in the STR (trial 18) at days (a) 0, (b) 1, (c) 3, (d) 4, (e) 5

Setting the speed of agitation to 75 rpm and initial aeration rate to 0.02 vvm led to the highest biomass of 11.8 g DW/3L on day 5 (trial 13), and 13.1 g/3L DW on day 8 (trial 15) representing a 4.52-fold and 5.02-fold higher respectively, in biomass from the initial inoculation. The biomass growth stopped on day 5 (trial 13) and day 8 (trial 15) as shown using FDA as a cell viability stain. The biomass concentrations reached in the bioreactor are

comparable to other studies: 2.3 g DW/L (278) and 9.8 g/L (258) DW, 50 g/L FW (5th day) (1) that is comparable with the fresh weight achieved in trial 4 (58 g/L FW), showing a good adaptation of the *V. labrusca* cv. Isabel CSC in bioreactor conditions.

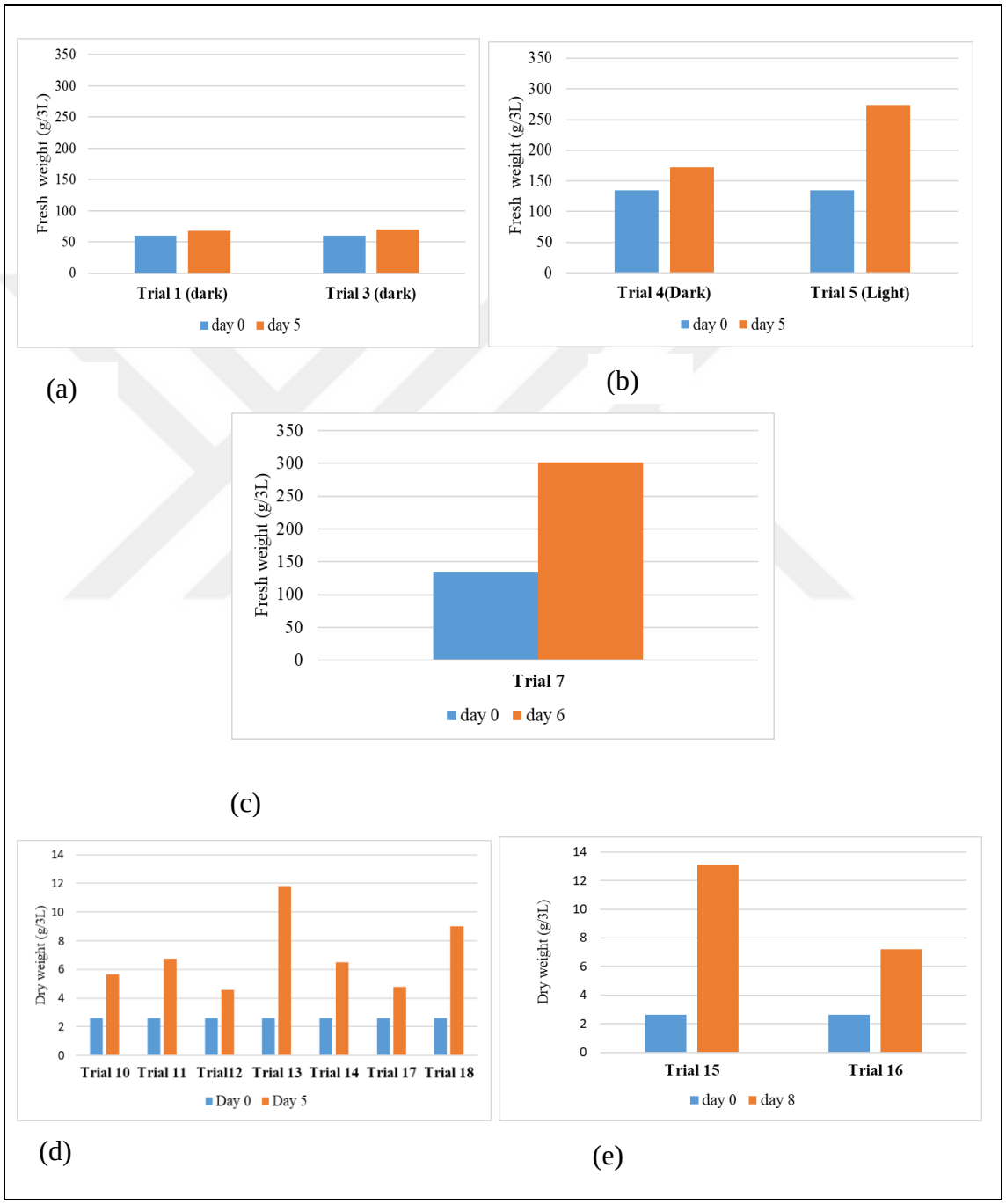


Figure 5.53. Growth profile of *V. labrusca* cv. Isabel cell biomass cultivated in STR. (a) trials 1 and 3, (b) trials 4 and 5 (Light), (c) trial 7 (Light), (d) trials 10-14 and trials 17-18, and (e) trials 15 and 16

5.10.2. Effect of casein hydrolysate on *V. labrusca* cv. Isabel cell growth

In the first and second experiments, we evaluated the effect of CH with presence/no presence of it on the media on biomass production. Samples of the *V. labrusca* cv. Isabel cells were not taken during the bioreactor run. The cells were harvested at the end of the run. This procedure was employed to eliminate a possible source of loss during sampling. Table 5.1 shows a summary of the increase in biomass for all experiments conducted in the STR and Erlenmeyer. We found that the used range of operating conditions was suitable for the cultivation of *V. labrusca* cv. Isabel CSC in the STR and Erlenmeyer. The highest-yielding bioreactions using CH in media which increased the biomass by 352.1% (5^{th} day), compared to the initial inoculum, were shown in trial 13, whereas the highest-yielding biorections without using CH in media which increased the biomass by 402.68 % (8^{th} day), compared to the initial inoculum, were shown in trial 15. Also, experiments conducted in Erlenmeyer without CH into media showed higher yielding of biomass by 623.43 % (8^{th} day) compared to the initial inoculum, whereas experiments conducted with CH into media compositions showed a yielding of biomass by 227 % (5^{th} day). Biomass yield on the bioreactor in each trial was shown in Table 5.2 whereas biomass yield on the shake flask for two species is shown in Table 5.3. The highest biomass yield in experiments with CH in media was achieved in trial 13 (0.27 g/g), whereas the highest biomass yield in the experiment without CH was achieved in trial 15 (0.34 g/g). In a shake flask, the biomass yield is higher than in a bioreactor.

No article has studied the behavior of *V. vinifera* or *V. labrusca* varieties CSC in a bioreactor in the presence or non-presence of CH. However, in comparison with the article (210) where *V. vinifera* cv. Gamay and *V.labrusca* cv. Concord CSC was cultivated in a shake flask in both media with and without CH, the data showed that *V. vinifera* cv. Gamay CSC has grown well in both media with and without CH presence which in many tissue culture systems is a good source of reduced nitrogen. On the other hand, *V. labrusca* cv. Concord CSC could only be cultivated in the non-presence of CH. CH includes 20 amino acids, amidst which, the L-alanine was found to trigger a programmed cell death response in *V. labrusca* cv. Concord cells. The coordinated activation of the genes for stilbene synthase (STS), cinnamic acid 4-hydroxylase (C4H), and phenylalanine ammonia-lyase (PAL) as well as the accumulation of stilbenes and other phenolic compounds were all associated with programmed cell death. In this article is found also that *V. vinifera* cv. Gamay callus could

be cultivated on both solid media with and without CH, whereas *V. labrusca* cv. Concord callus placed in media with CH did not grow but turned dark yellow to brown with time and died. In contrast with this article, results performed in this thesis show that *V. labrusca* cv. Isabel callus and suspension culture in the shake flask and bioreactor was cultivated successfully in both media with and without CH, whereas *V. vinifera* cv. Öküzgözü callus was successfully cultivated in the media with CH and *V. vinifera* cv. Öküzgözü CSC could only be established in the media without CH which is in contrast with the article (177) where *V. vinifera* cv. Öküzgözü callus was cultivated in the media with the absence of CH and *V. vinifera* cv. Öküzgözü CSC was cultivated in the media with the presence of CH.

Table 5.1. Yields of *V. labrusca* cv. Isabel Bioreactor culture.

Experiment (5^{th} day)	IFW (g DW)	FFW (g DW)	% Biomass increase
Trial 18	2.61	9	244.8
Trial 17	2.61	4.768	82.68
Trial 16	2.61	7.22	193.48 (8^{th} day)
Trial 15	2.61	13.12	402.68 (8^{th} day)
Trial 14	2.61	6.5	149
Trial 13	2.61	11.8	352.1
Trial 12	2.61	4.56	74.7
Trial 11	2.61	6.745	158.23
Trial 10	2.61	5.64	116.09
Shake Flask (First study)	1.71	2,985	74.56
Shake Flask (Second study)	1.92	6.28	227
Shake Flask (Third study) No CH	1.92	13.8	623.43 (8^{th} day)

IFW (g): initial fresh weight of inoculum in grams; FFW (g) final fresh weight of inoculum in grams; % biomass increase: [{(FFW-IFW)/IFW}*100].

Table 5.2. Biomass yield in *V.labrusca* cv. Isabel cultivated in STR.

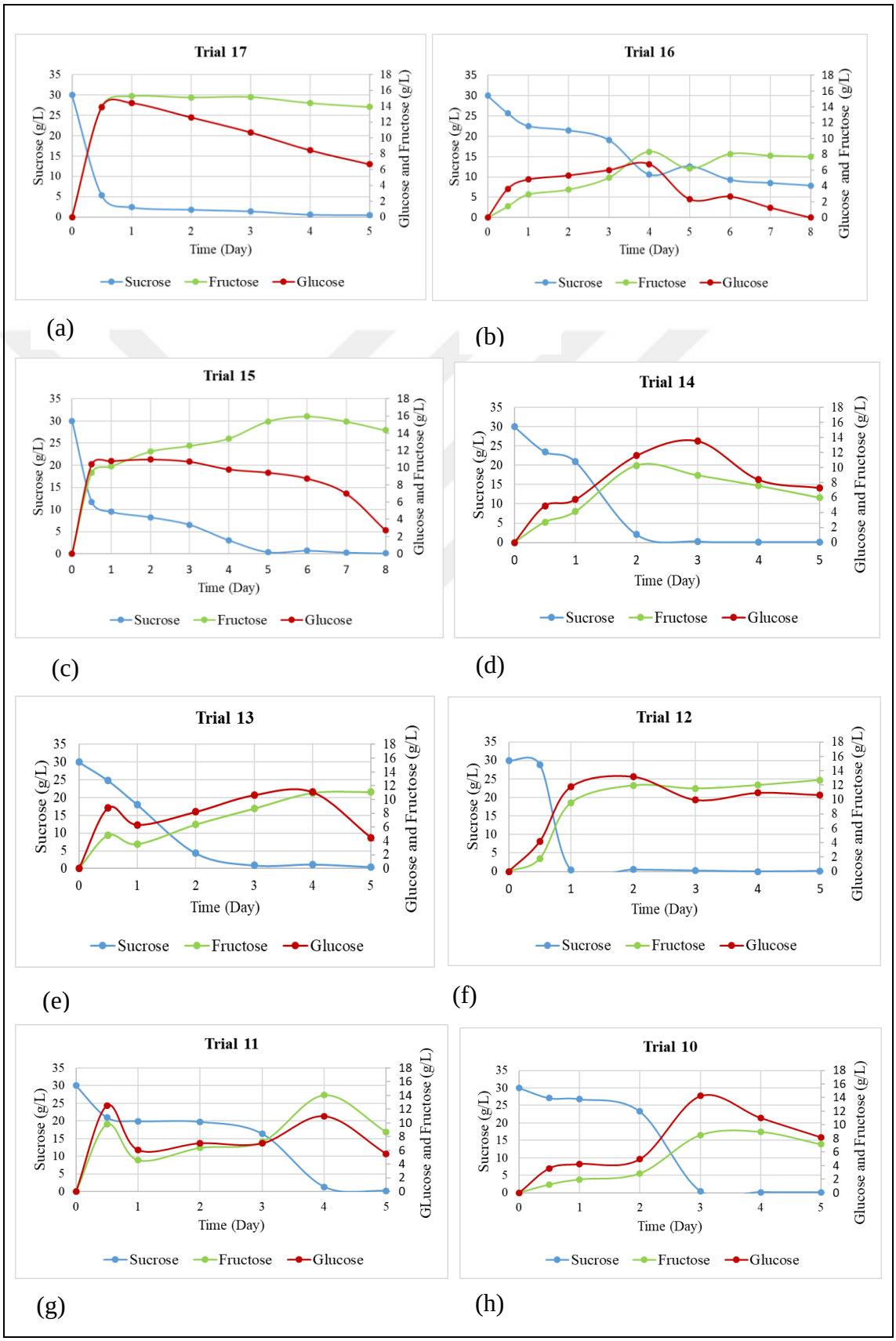
Trial	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**10**
Consumed sugar (g/L)	9.66	14.47	12.92	16.77	14.44	6.71	16.06	14.67

Biomass (g DW/L)	1.589	2.4	4.37	2.166	3.93	1.53	2.24	1.88
Yx/s (g/g)	0.164	0.166	0.34	0.13	0.27	0.22	0.14	0.13

Table 5.3. Biomass yield in *V. labrusca* cv. Isabel and *V. vinifera* cv. Öküzgözü cultivated in shake flask.

Erlenmeyer	***V. labrusca* cv. Isabel (First study) (5th day)**	***V.labrusca* cv. Isabel (Second study) (5th day)**	***V. labrusca* cv. Isabel (without CH) (8th day)**	***V. vinifera* cv. Öküzgözü (5th day)**
Consumed sugar (g/L)	1.68	9.08	19.88	4.3
Biomass (g DW /L)	2.95	6.28	13.89	4.8
Yx/s (g/g)	1.75	0.69	0.69	1.1

5.10.3. Sugar analysis of *V. labrusca* cv. Isabel cell suspension culture cultivated in STR



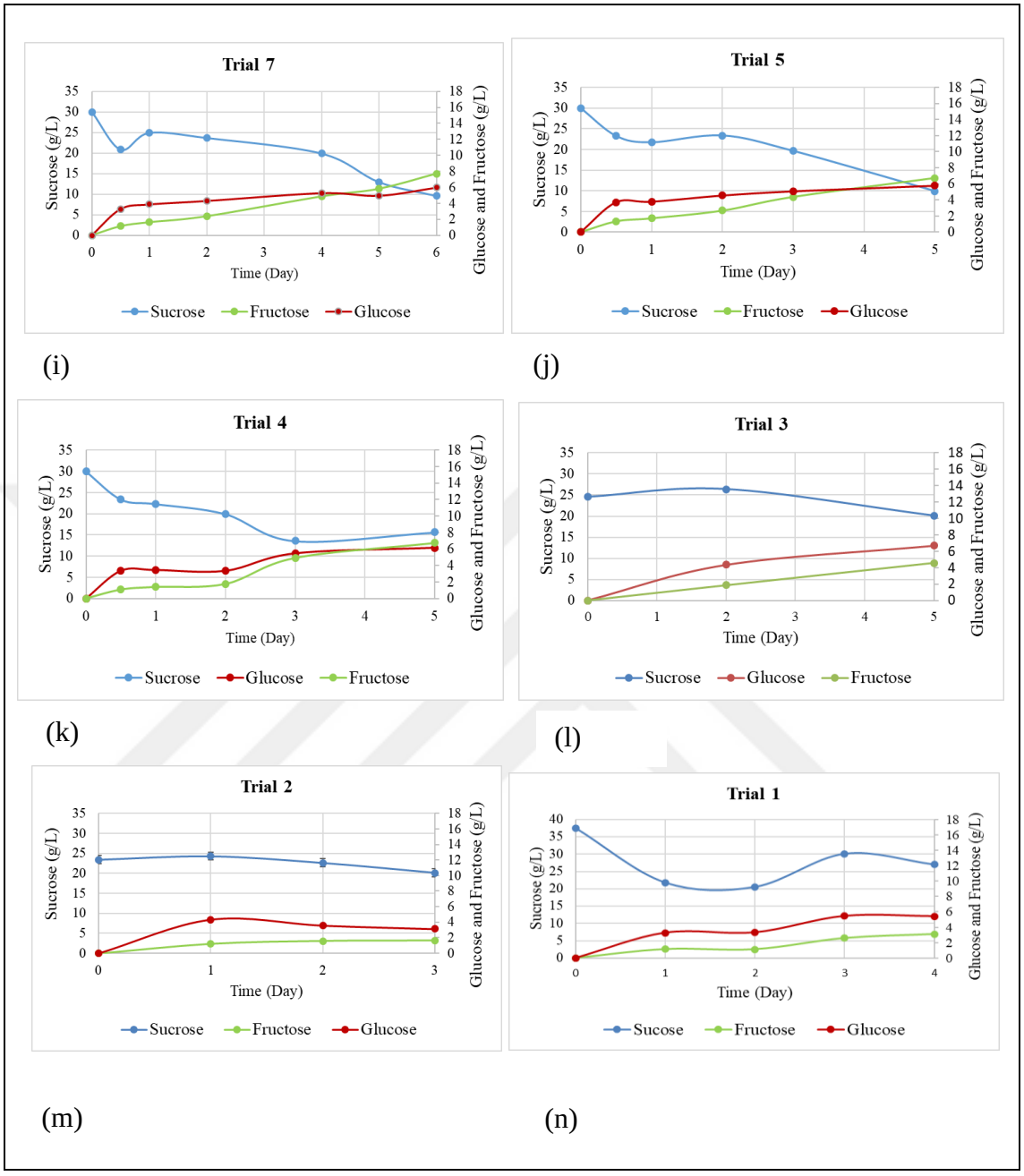


Figure 5.54. Sugar anaysis of *V. labrusca* cv. Isabel CSC in STR. (a) trial 17, (b) trial 16, (c) trial 15, (d) trial 14, (e) trial 13, (f) trial 12, (g) trial 11, (h) trial 10, (i) trial 7, (j) trial 5, (k) trial 4, (l) trial 3, (m) trial 2, and (n) trial 1

5.11. EXTRACELLULAR TRANS-RESVERATROL EXTRACTION FROM *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE IN STR

Extracellular t-resveratrol was identified and quantified by comparison with the authentic standard of 98 % purity resveratrol based on its retention time. Under these HPLC conditions, the retention time of resveratrol was 16.745 min. The calibration curve was obtained from the increases in the peak areas of standard t-resveratrol which was observed at minutes 16.745 min (Figure 5.55). The X-axis intercepts were taken into account for t-resveratrol amount determination. The calibration curve of standard t-resveratrol is shown in Figure 5.56.

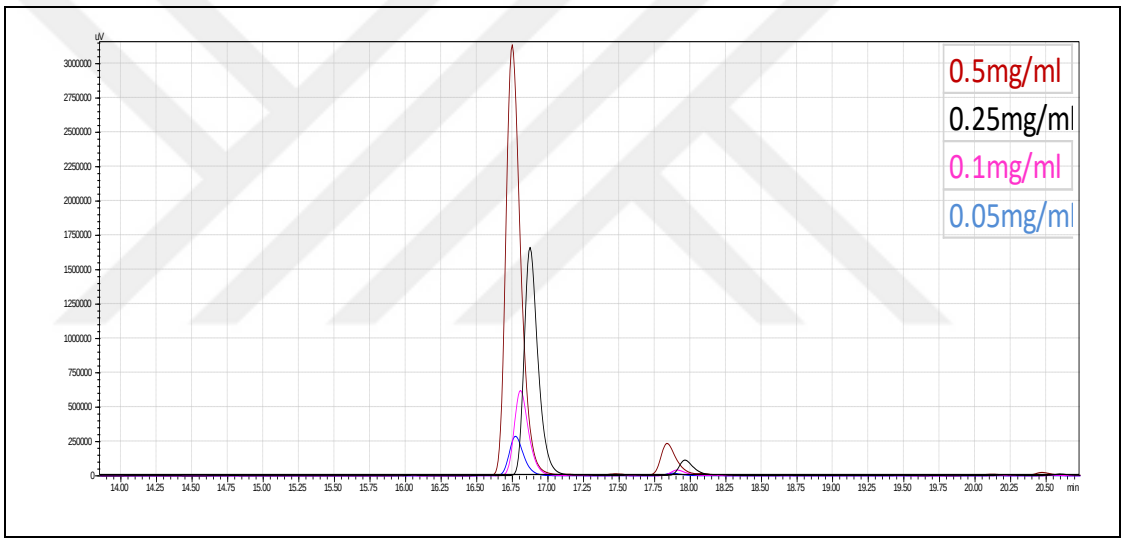


Figure 5.55. HPLC chromatogram of standard t-resveratrol for *Vitis labrusca* cv. Isabel in STR.

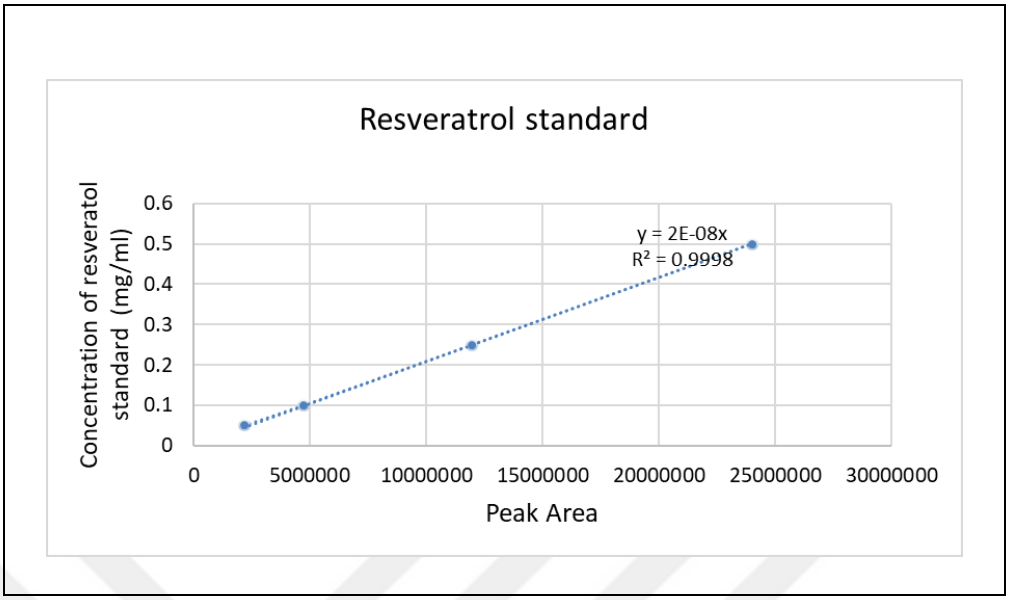
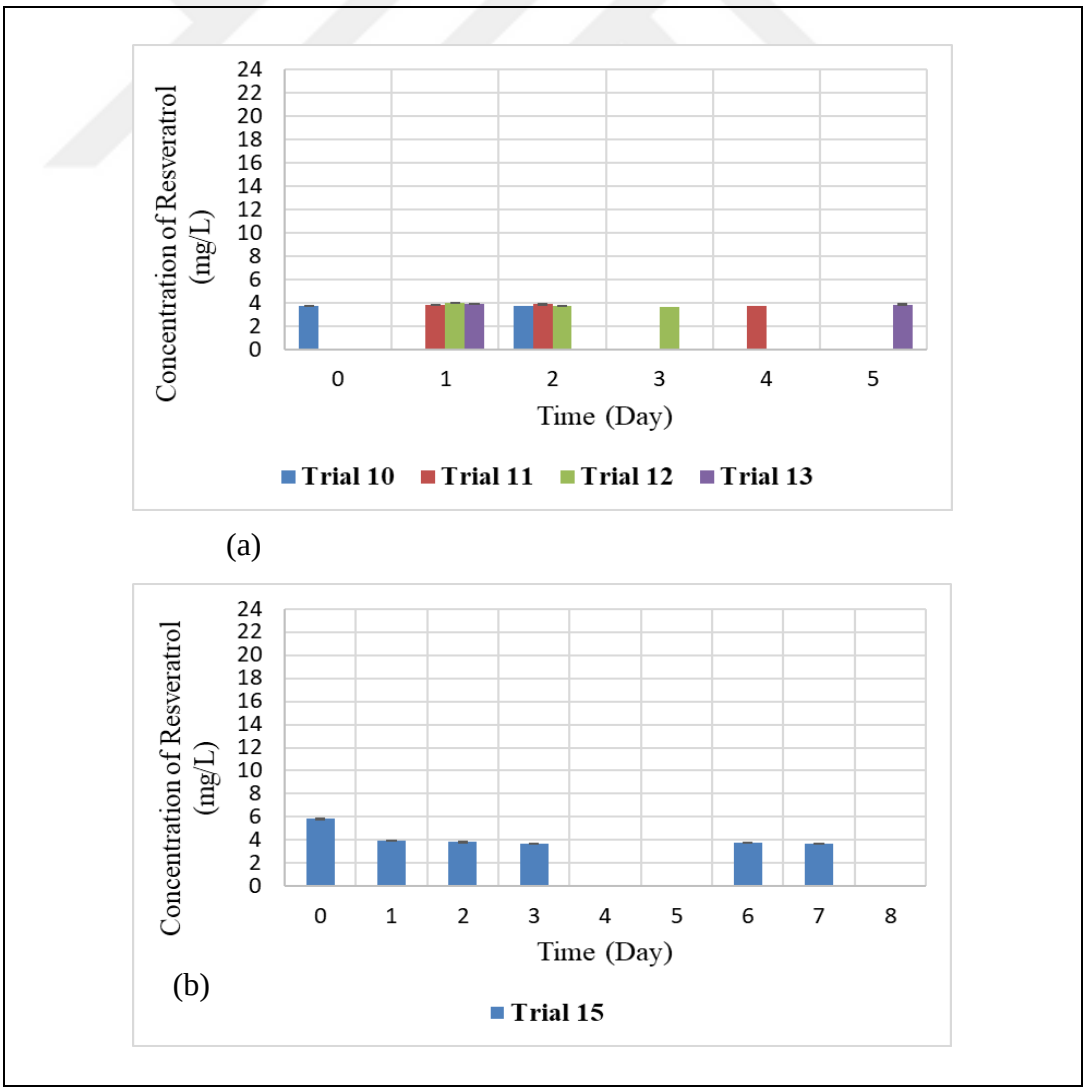


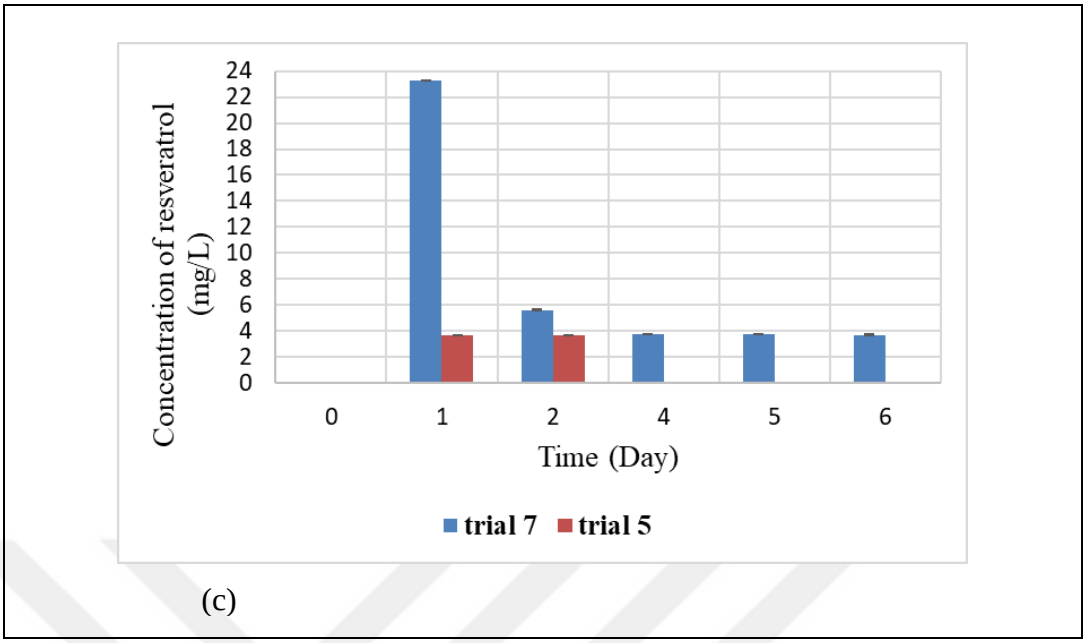
Figure 5.56. The calibration curve of standard t-resveratrol for *Vitis labrusca* cv. Isabel in STR

In control samples of *V. labrusca* cv. Isabel CSC cultivated in an STR, the extracellular t-resveratrol was found around 4 mg/L in all trials conducted in dark (trial 10-trial 13) (Figure 5.57 a). t-resveratrol was found also in control samples of *V. labrusca* cv. Isabel cultivated in media without the presence of CH which was around 6 mg/L (trial 15) (Figure 5.57 b). In trial 7 conducted under light conditions, t-resveratrol was found 23.26 mg/L (Figure 5.57 c). The results showed that the light condition induced t-resveratrol accumulation. Figure 5.58 represent an HPLC chromatogram showing the peaks of extracellular t-resveratrol production in *V. labrusca* cv. Isabel cultivated in the bioreactor. Light is a physical elicitor that can induce SMs production. Accumulation of t-resveratrol in response to light was shown in articles (279) (280) but little is known about how light affects stilbene content in grapevine cell culture, and the most of research on UV light effect has been focused on increasing the stilbene content in grape berries (281)(282)(283), leaves (284), and callus tissue (185) (285) (286). Furthermore, only calluses that were actively growing were able to produce stilbenes (including trans-resveratrol), according to Keller et al (2000) (286). The results presented here provide useful information about the use of light to increase the contents of t-resveratrol in *Vitis* plant CSC. The results in the bioreactor show the accumulation of a high amount of biomass (trial 5 and trial 7) and t-resveratrol in presence of light (trial 7) compared to the samples conducted in the dark unlike the research found about the *V. vinifera* cv. Shahani cell growth and phenolic compounds accumulation in shake

flask where no significant differences of FW and DW were found between cells cultivated in the dark and light. On the other hand, cells cultivated under dark conditions were more capable than those grown in light in terms of t- resveratrol production (287). Also, article (177) studied the effect of light/dark in terms of t-resveratrol production (data shown in table 2.4) where the content of t-resveratrol was higher on the cells incubated at dark conditions. t-Resveratrol can be found in the cis or trans isomers. Its trans isomer is found in plants, but low amount of cis isomer has been detected in red wines. After exposure to the UV and high white light, the trans form may change to cis form (177).

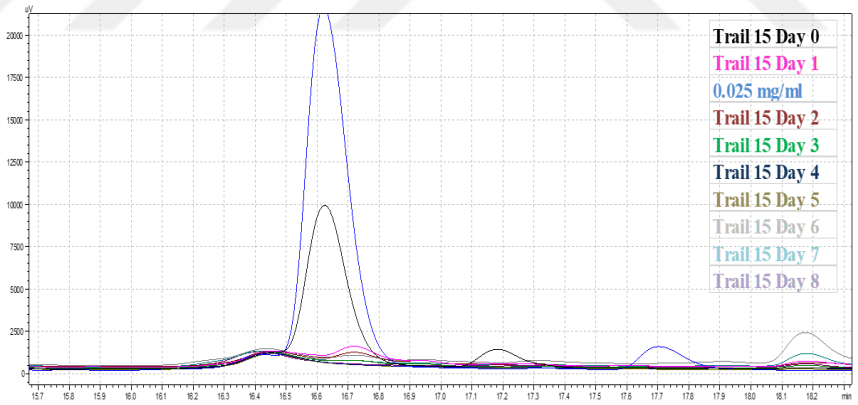
Unlike the results performed in many articles using *V. labrusca* cv. Concord and *V. vinifera* varieties where have shown no t-resveratrol production without elicitation in the shake flask and the bioreactor (1) (258) (260), this thesis shows the opposite where t-resveratrol is found in the shake flask and each trial performed in STR without elicitors (trial 10-13) (Figure 5.57a).



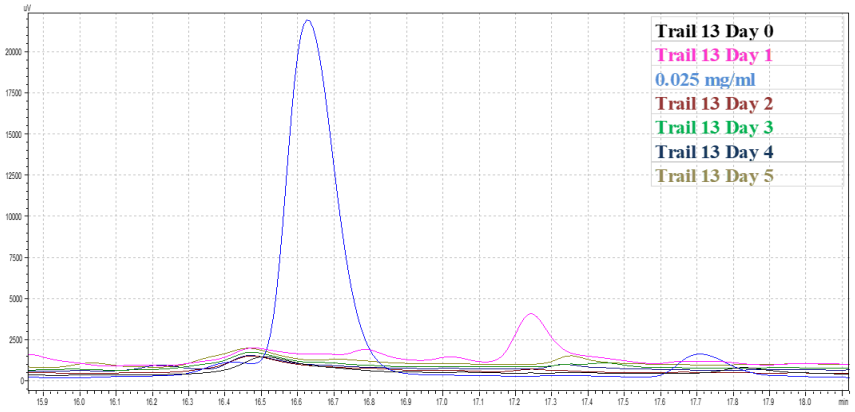


(c)

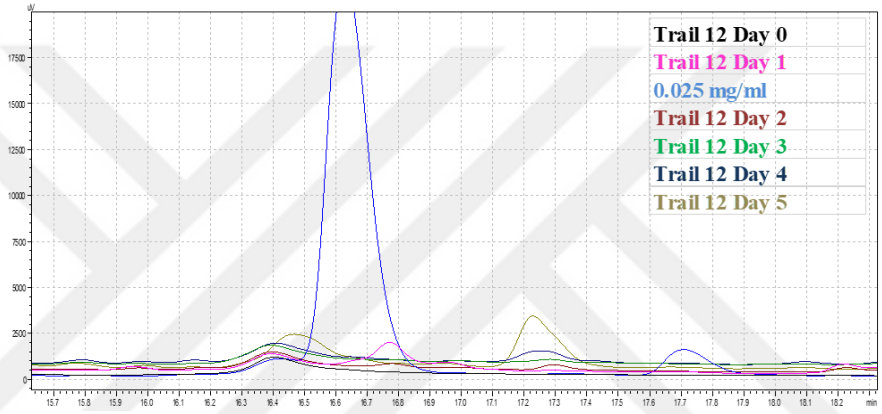
Figure 5.57. Extracellular t-resveratrol production from *V. labrusca* cv. Isabel CSC cultivated in the STR. (a) trials 10-13, (b) trial 15, and (c) trials 5 and 7



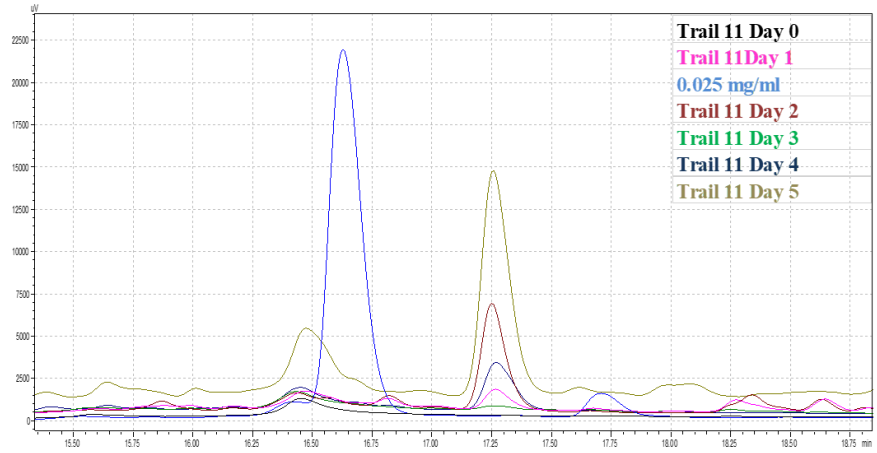
(a)


Trail 13 Day 0
Trail 13 Day 1
0.025 mg/ml
Trail 13 Day 2
Trail 13 Day 3
Trail 13 Day 4
Trail 13 Day 5

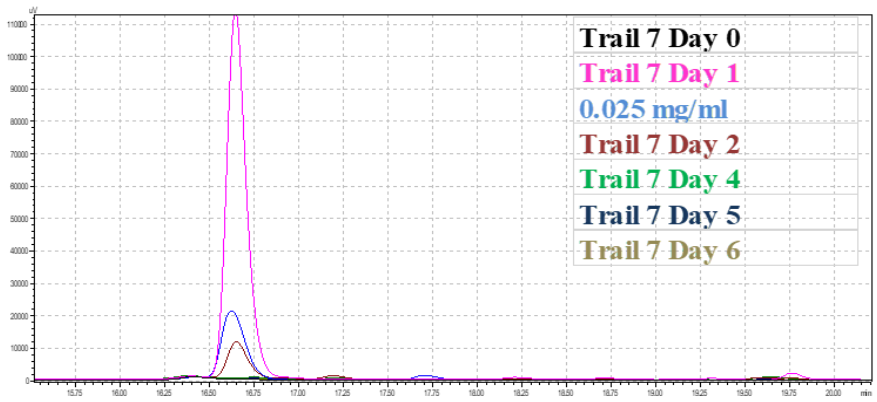
(b)


Trail 12 Day 0
Trail 12 Day 1
0.025 mg/ml
Trail 12 Day 2
Trail 12 Day 3
Trail 12 Day 4
Trail 12 Day 5

(c)


Trail 11 Day 0
Trail 11Day 1
0.025 mg/ml
Trail 11 Day 2
Trail 11 Day 3
Trail 11 Day 4
Trail 11 Day 5

(d)



(e)

Figure 5.58. HPLC chromatograms of extracellular t-resveratrol from *V. labrusca* cv. Isabel CSC cultivated in the STR. (a) trial 15, (b) trial 13, (c) trial 12, (d) trial 11, and (e) trial 7

5.12. ANTHOCYANIN EXTRACTION FROM *V. LABRUSCA* CV. ISABEL CELL SUSPENSION CULTURE CULTIVATED IN ERLENMEYER AND BIOREACTOR

These experiments aimed to investigate the effect of light on the anthocyanin production that was present in the CSC cultivated in Erlenmeyer and the STR (trials 5 and 7). The concentration of anthocyanins was quantified as described by Hirasuna *et al*. 1991. Anthocyanin was calculated using the following formula that was formulated by Lee *et al*. 2005 (shown in the Material and Method part). Anthocyanin production was similar to that found in cells treated in dark and light conditions and the presence of light had no significant effect on total anthocyanin production in *V. labrusca* cv. Isabel CSC cultivated in STR and Erlenmeyer (Figure 5.59 a-e). Anthocyanin biosynthesis was stimulated in both light and dark conditions in the article (177). In light conditions, anthocyanin was found to be 1.584 CV/g in Gamay, 1.893 CV/g in Kalecik karası, and 2.667 CV/g in Öküzgözü. On the other hand, in dark conditions, it was found to be 1.06 CV/g in Gamay, 2.49 CV/g in Kalecik karası, and 2.493 CV/g in Öküzgözü. This is comparable to our results.

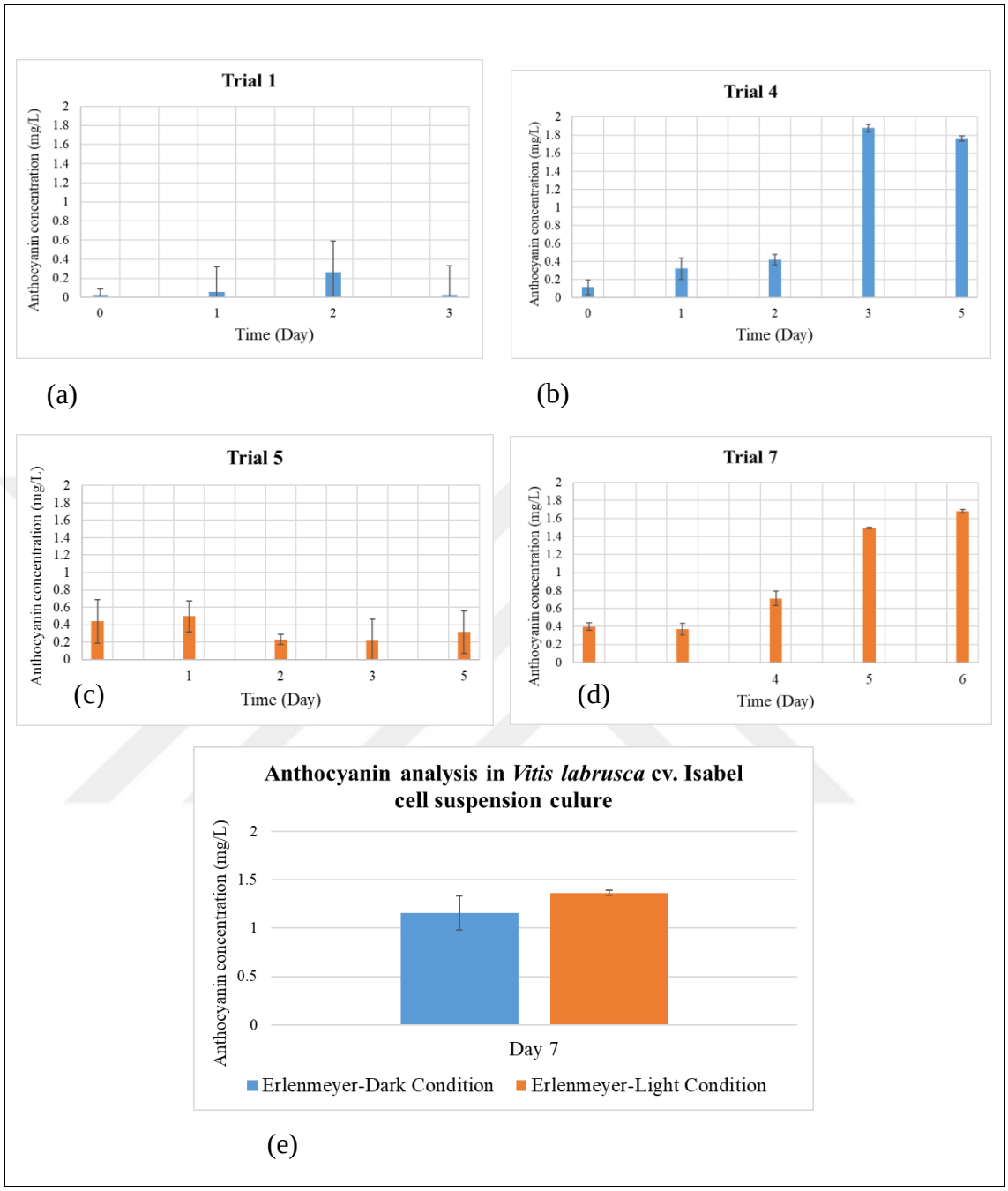
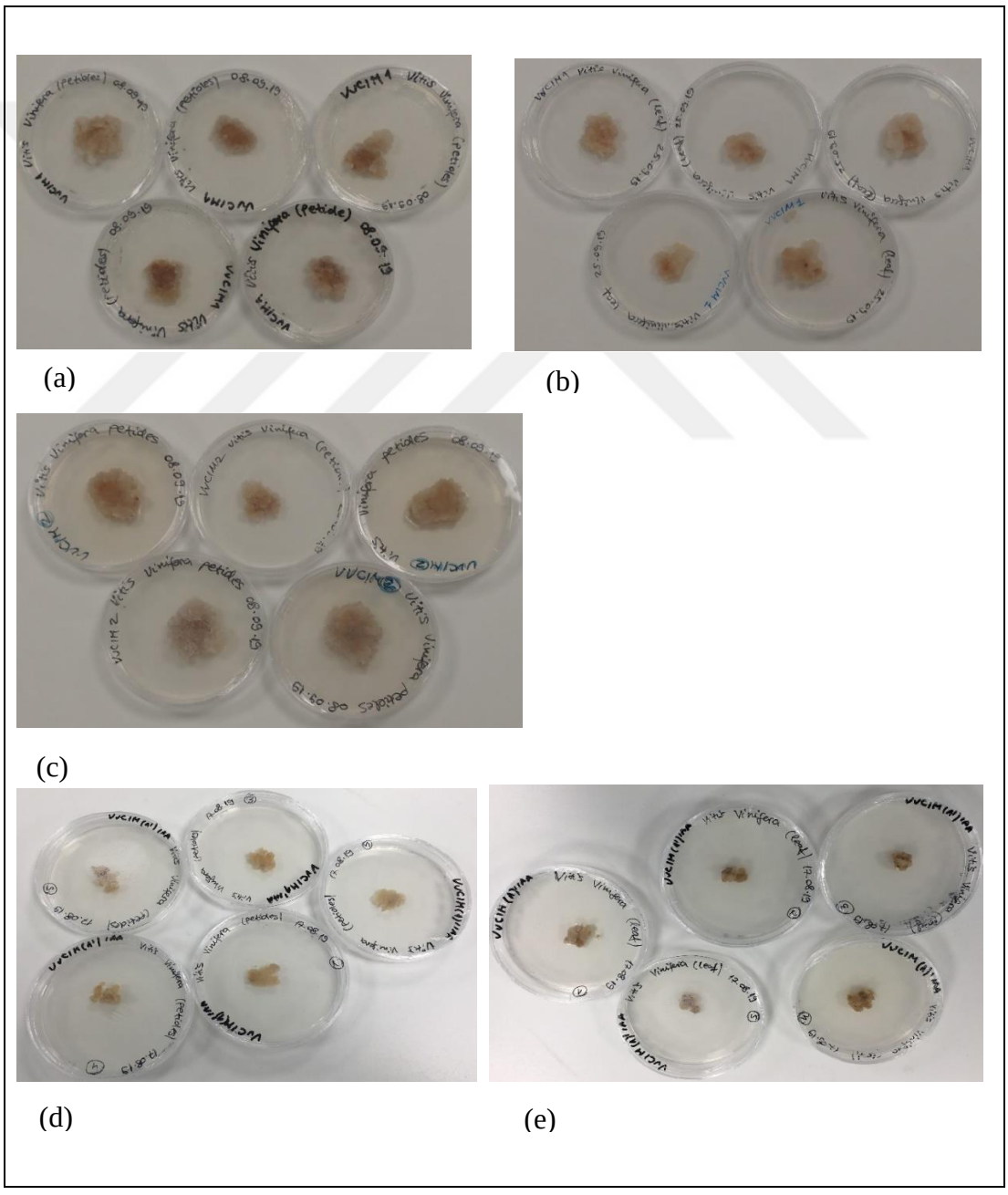


Figure 5.59. Anthocyanin analysis in *V. labrusca* cv. Isabel CSC. (a) Trial 1 (Dark), (b) Trial 4 (Dark), (c)Trial 5 (Light), (d) Trial 7 (Light), and (e) Erlenmeyer

***VITIS VINIFERA* (L.) CV. ÖKÜZGÖZÜ**

5.13. CALLUS INDUCTION OF *V. VINIFERA* CV. ÖKÜZGÖZÜ

After 1 month, calli were separated from the explants and subcultured to fresh media. MS and B5 medium without plant growth regulator failed to induce the callus formation. Also, no callus formation was found in VvCIM5, VvCIM6, and VvCIM7 media (Figure 5.60).



(a) (b)

(c)

(d) (e)

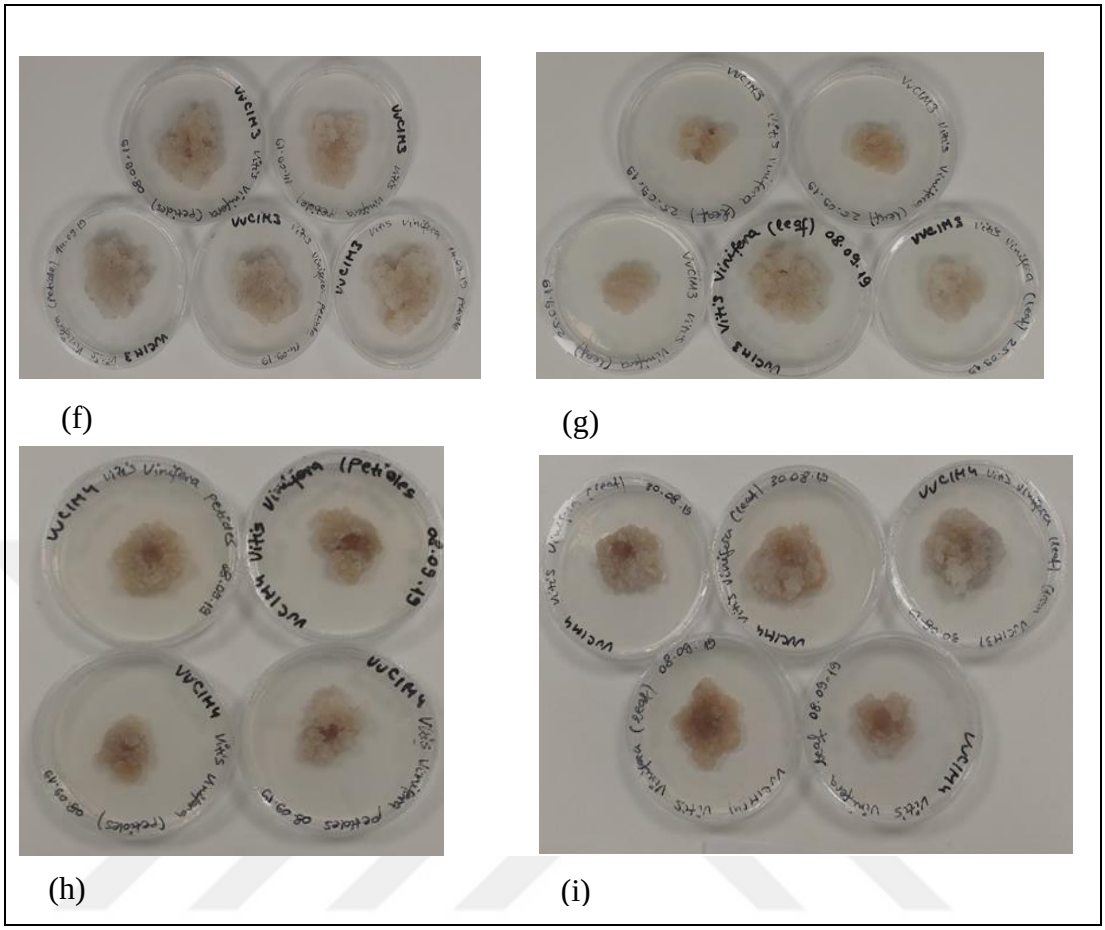


Figure 5.60. Callus induction from petiole and leaf explant of *V. vinifera* cv. Öküzgözü. (a) *V. vinifera* cv. Öküzgözü callus from petiole explant into VvCIM1 media, (b) *V. vinifera* cv. Öküzgözü callus from leaf explant into VvCIM1 media, (c) *V. vinifera* cv. Öküzgözü callus from petiole explant into VvCIM(2), (d) *V. vinifera* cv. Öküzgözü callus from petiole explant into VvCIM(1)$^{'IAA}$, (e) *V. vinifera* cv. Öküzgözü callus from leaf explant into VvCIM(1)$^{'IAA}$, (f) *V. vinifera* cv. Öküzgözü callus from petiole explant into VvCIM(3), (g) *V. vinifera* cv. Öküzgözü callus leaf explant into VvCIM(3), (h) *V. vinifera* cv. Öküzgözü callus from petiole explant into VvCIM(4), and (i) *V. vinifera* cv. Öküzgözü callus from leaf explant into VvCIM(4)

5.13.1. Fresh weight of *V. vinifera* cv. Öküzgözü callus

The highest FW of *V. vinifera* cv. Öküzgözü callus obtained from both petiole and leaf explant was observed in VvCIM3 media compositions containing 3.164 g/L B5 media, 20 g/L sucrose, 250 mg/L CH, 1 mg/L NAA and 0.2 mg/L kinetin (Figure 5.62 g, h). For callus

obtained into VvCIM3 from petioles explant, the fresh weight on the 40th day was 12639,8 mg, whereas for callus obtained from leaves explant into VvCIM3 was 8532.2 mg on the 40th day (Figure 5.61a,b and Figure 5.62 g, h).

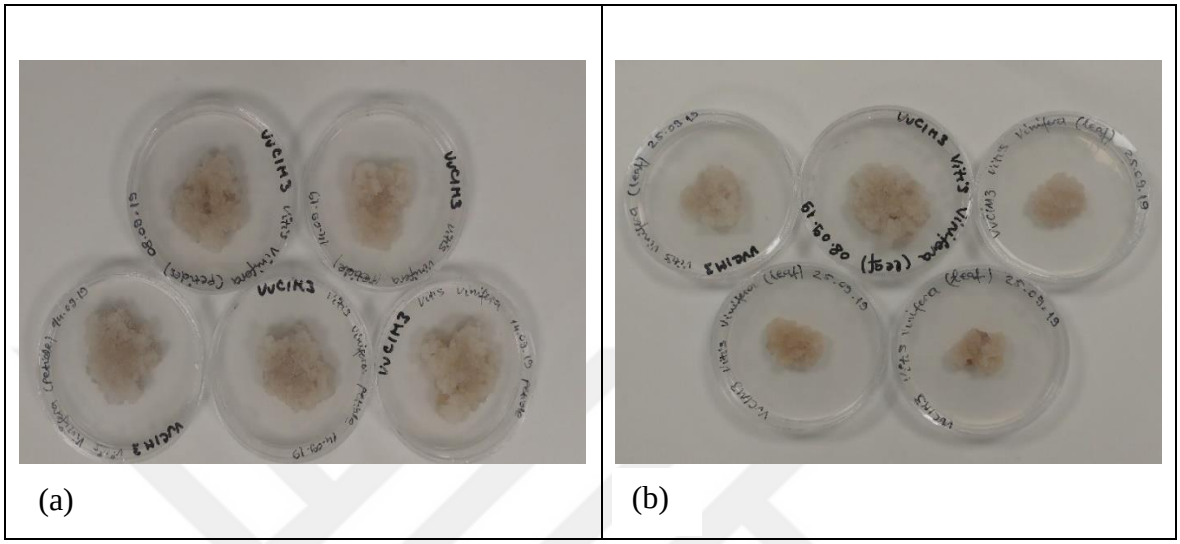
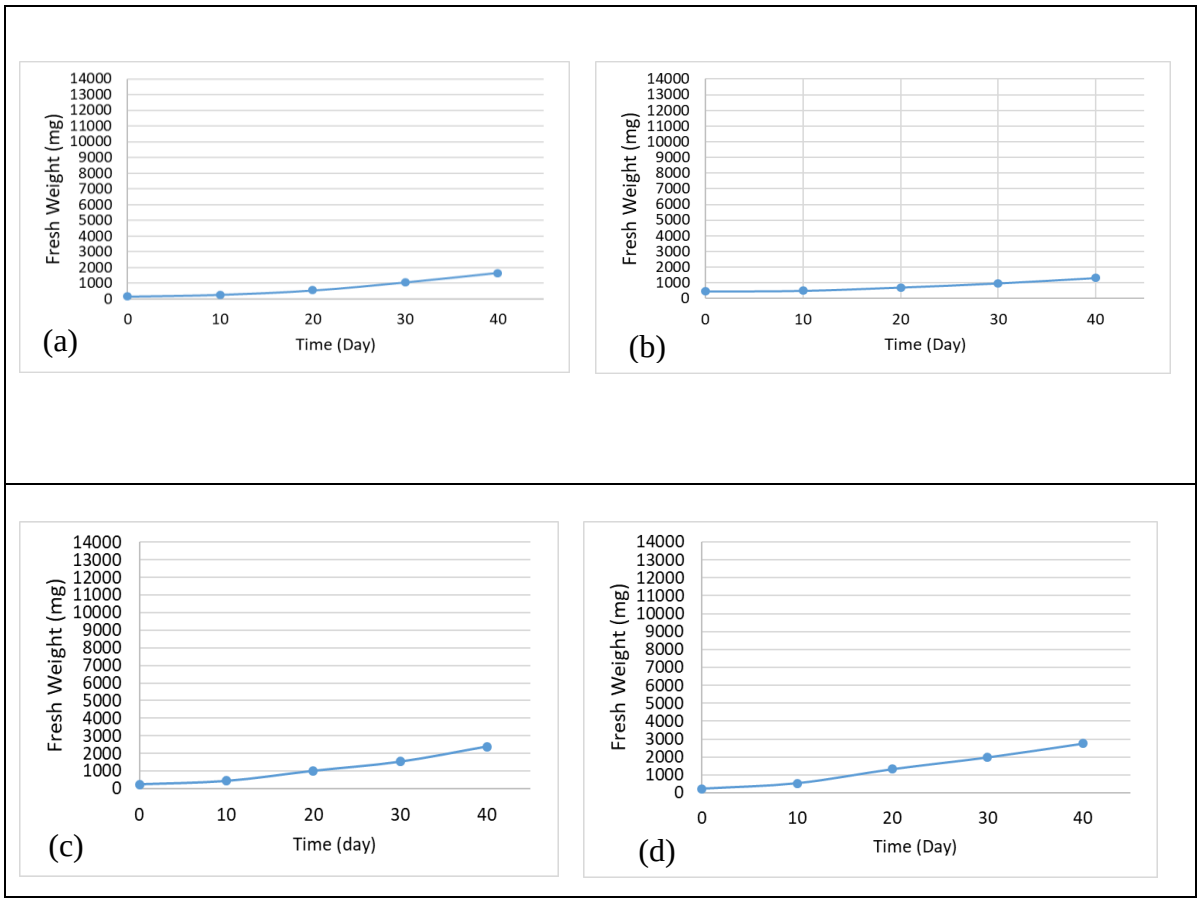


Figure 5.61. *V. vinifera* cv. Öküzgözü callus grown in VvCIM3 media. (a) callus from petiole, and (b) callus from leaves



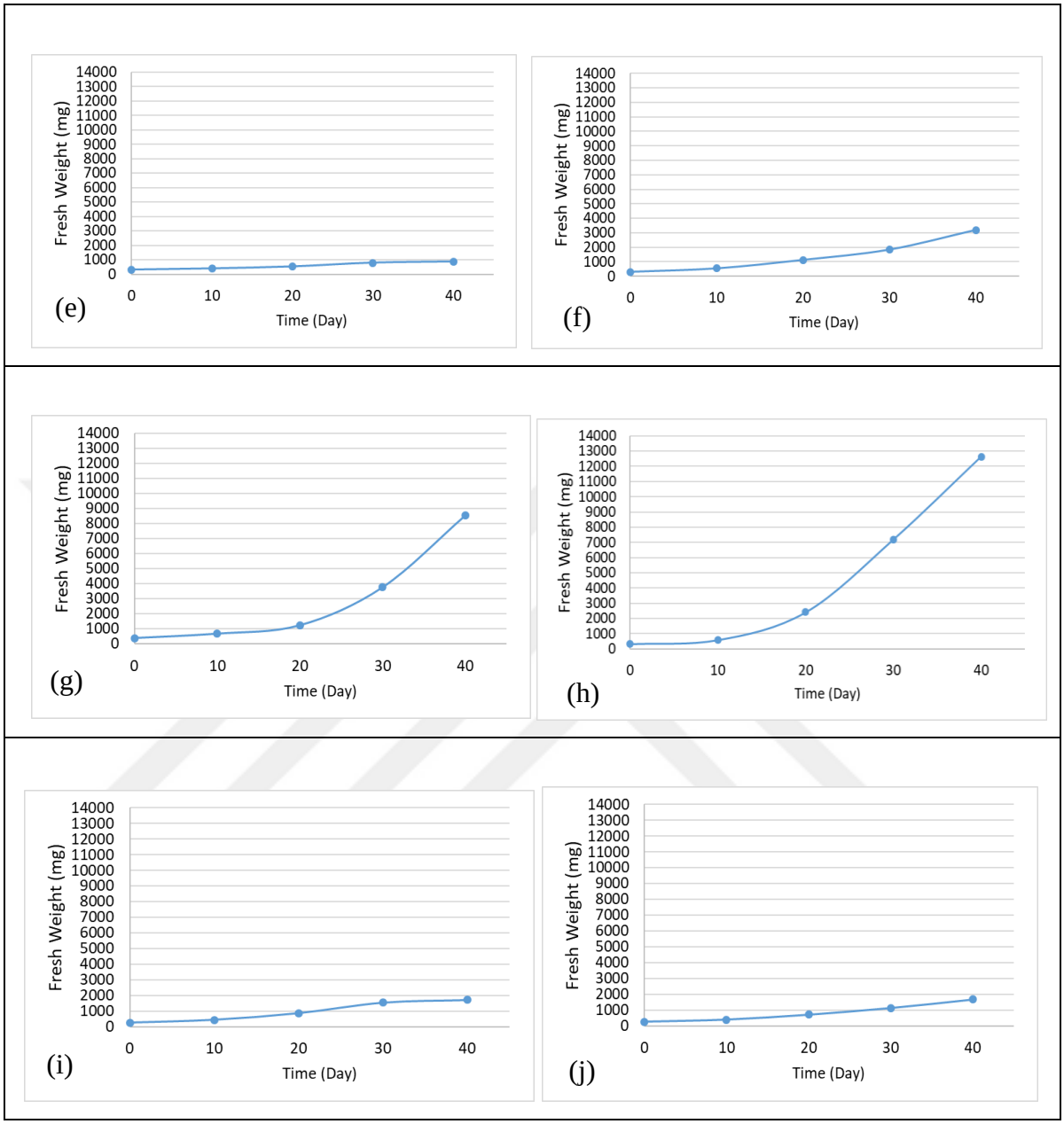


Figure 5.62. Growth profile of *V. vinifera* cv. Öküzgözü callus. (a) VvCIM1-leaf callus, (b) VvCIM1-petiole callus, (c) VvCIM(1)IAA'-leaf callus, (d) VvCIM(1)IAA'-petiole callus, (e) VvCIM2-leaf callus, (f) VvCIM2-petiole callus, (g) VvCIM3-leaf callus, (h) VvCIM3-petiole callus, (i) VvCIM4-leaf callus, and (j) VvCIM4-petiole callus

5.13.2. Assessment of growth kinetics for *V. vinifera* cv. Öküzgözü callus

The calli data of CRGR day^{-1} is illustrated in Table 5.63 a, b. Callus formation from petioles showed the highest maximum growth rate after 15 days (0.141 day^{-1}) obtained into VvCIM3

(Figure 5.63 a), whereas callus formation from leaf explants showed the highest maximum growth rate after 35 days (0.164 day^{-1}) obtained in VvCIM3 media (Figure 5.63 b). The lowest maximum growth rate of callus obtained from petioles was observed in VvCIM1 media (0.06 day^{-1}), whereas the lowest maximum growth rate of callus obtained from leaves was observed in VvCIM2 (0.04 day^{-1}). Also, VvCIM3 media showed the best results for relative fresh weight measurement (CRFW). CRFW was 3.09, the highest for calli obtained from petioles and CRFW was 2.07, the highest for calli obtained from leaves (Figure 5.63 c,d).

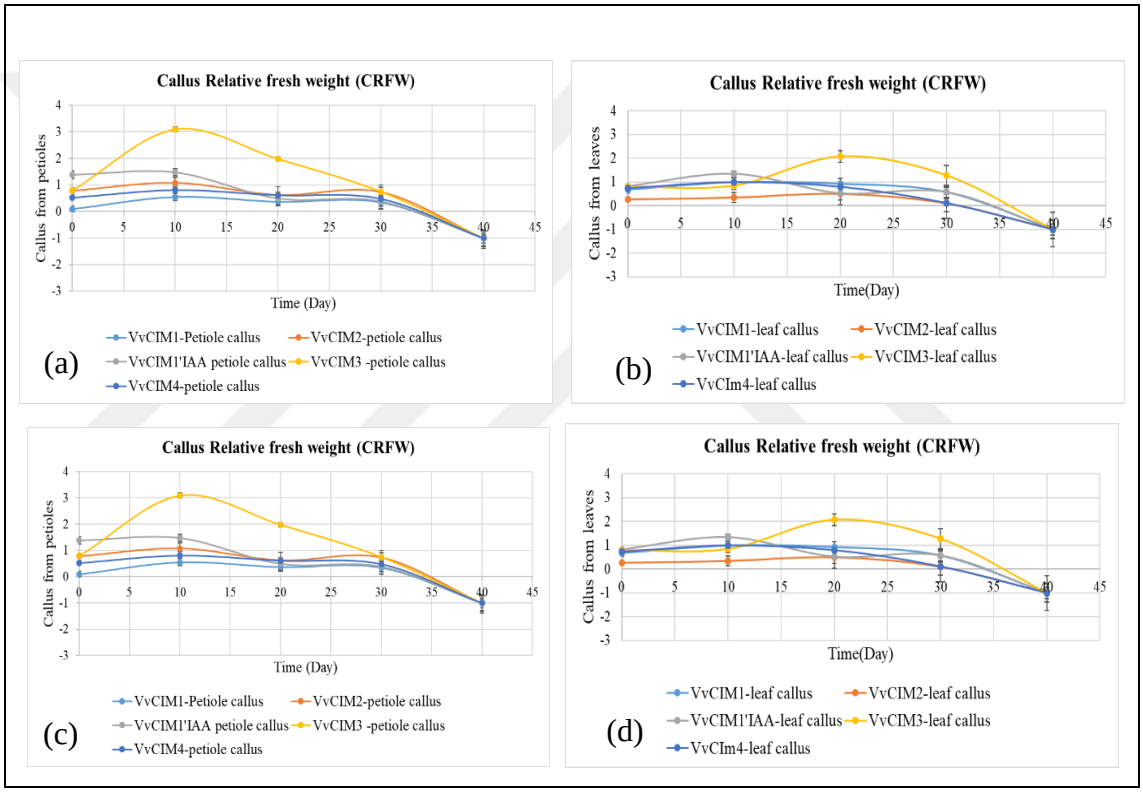


Figure 5.63. (a) Callus relative growth rate (CRGR) obtained from petioles, (b) Callus relative growth rate (CRGR) obtained from leaves, (c) Callus relative fresh weight (CRFW) obtained from petioles, and (d) Callus relative fresh weight (CRFW) obtained from leaves

Callus growth percentages were calculated for each medium. As Figure 5.64 a,b show, the highest percentage of calli obtained from petioles was found in VvCIM3 media (97.4 %), and the highest percentage of calli obtained from leaves was found in VvCIM3 (95.6 %). The lowest percentage of calli growth obtained from petioles was found in VvCIM1 (65 %),

whereas the lowest percentage of callus growth obtained from leaves was found in VvCIM2 (64.5 %).

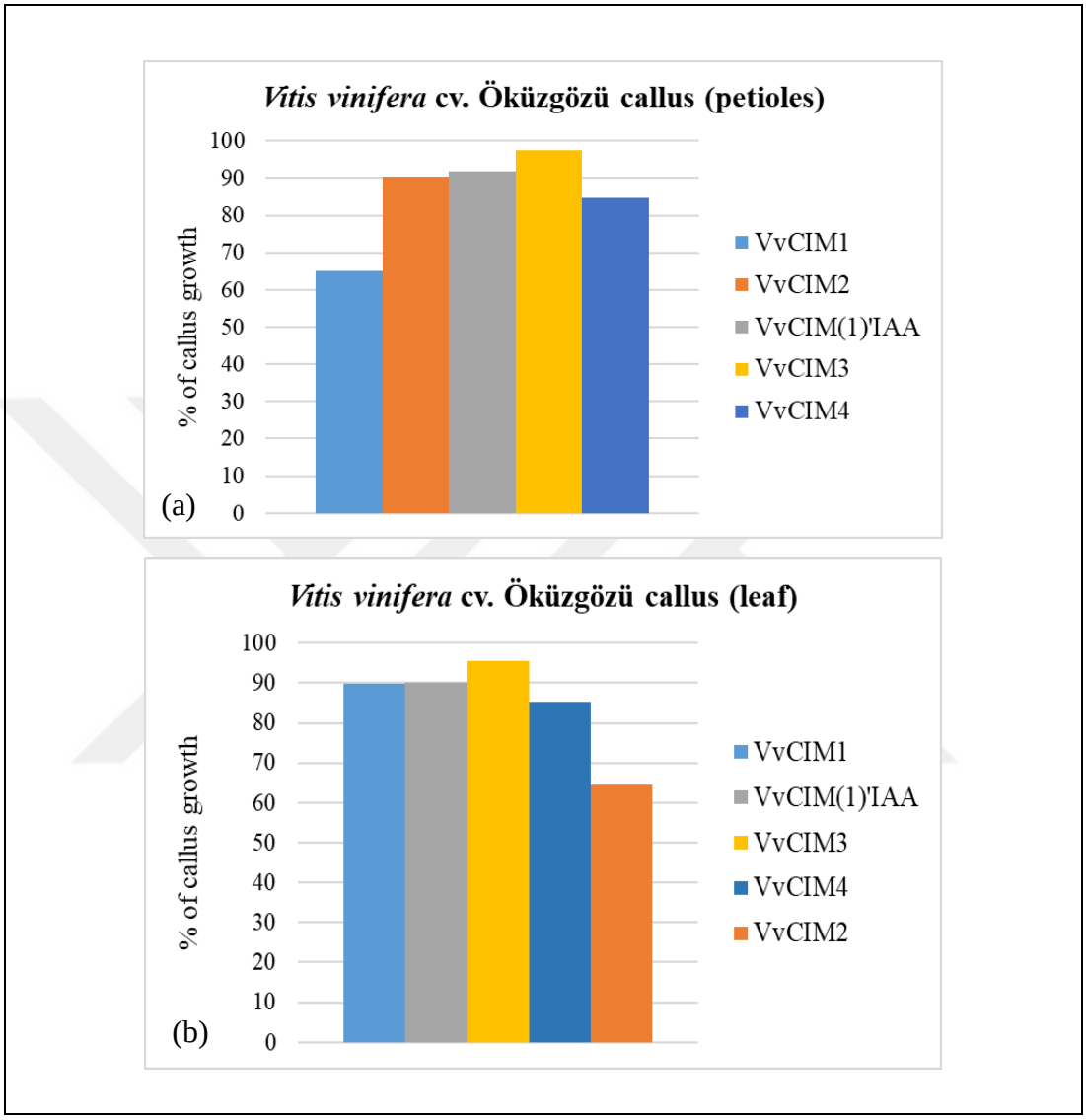


Figure 5.64. Percentage of *V. vinifera* cv. Öküzgözü callus growth to five media composition. (a) callus from petioles, and (b) callus from leaves

5.14. ESTABLISHMENT OF *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

VvSCIM13 media has shown the best performance of *V. vinifera* cv. Öküzgözü CSC growing (Figure 5.65 a, b). Other media compositions used did not work for *V. vinifera* cv Öküzgözü CSC. *V. vinifera* cv. Öküzgözü CSC has a darker color when compared to *V.*

labrusca cv. Isabel cells, however, the same case is also found in callus induction media which might indicate that this is a characteristic of *V. vinifera* cv. Öküzgözü.

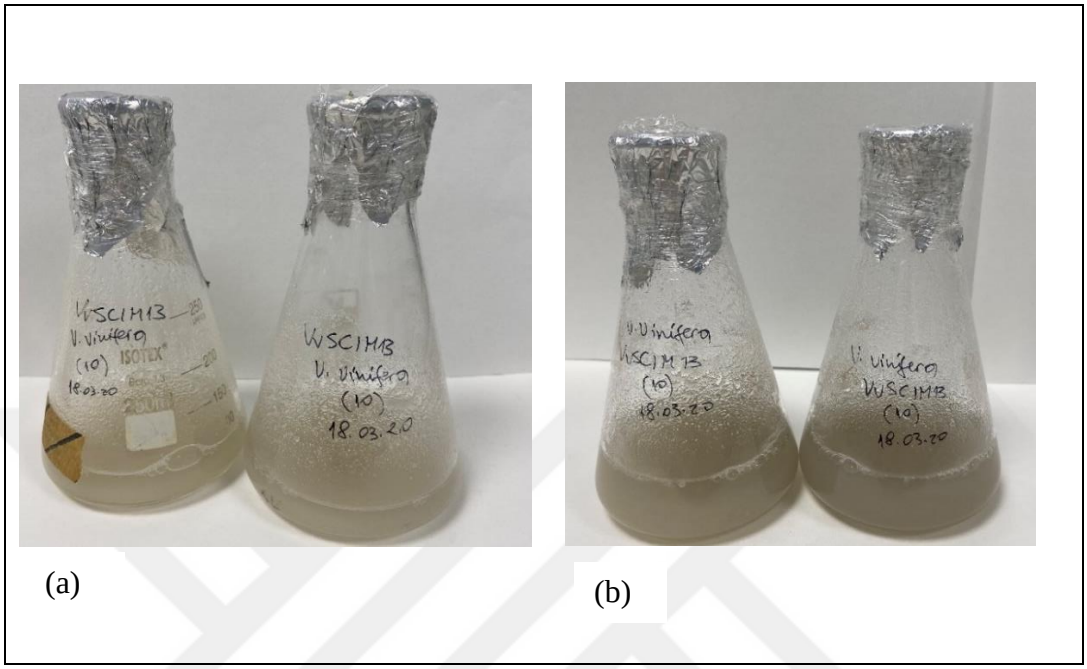


Figure 5.65. *V. vinifera* cv. Öküzgözü CSC cultivated. (a) 50 ml VvSCIM13 media, and (b) 100 ml VvSCIM13 media

5.15. DETERMINATION OF CELL GROWTH (FW AND DW) AND PH VALUE FOR *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

For *V. vinifera* cv. Öküzgözü cells, FW on 1st day was 43.57 g/L whereas FW on the 13th day was 276.9 g/L. On the other hand, the DW increased from 2.11 g/on the 1st day up to 10.91 g/L on the 13th day (Figure 5.66 a). The biomass concentration reached in control cells of *V. vinifera* cv. Öküzgözü is comparable with other studies using different varieties of *V. vinifera* (L): 11 g DW/L (133), 10.9 g DW/L (124). *V. vinifera* cv. Öküzgözü cells displayed an exponential phase between day 5 and day 13. The doubling time of *V. vinifera* cv. Öküzgözü cells were 4.2 days. The ratio of FW/DW increased as sucrose concentration dropped in the media (Figure 5.66 b) (259). The maximum specific growth rate for *V. vinifera* cv. Öküzgözü cells was 0.21/day (Figure 5.66 c).

The pH profile shows an initial decrease during the lag phase pH =5.41 and an increasing trend during the exponential growth phase up to pH=6.56. The initial pH in most PCC ranges between 5.5-5.9. A rapid drop in pH happens due to the initial ammonium uptake and

acidification of media due to cell lysis. Then, pH increases after a few days and reached a stable level which is related to the uptake of nitrates (266) (Figure 5.66 a).

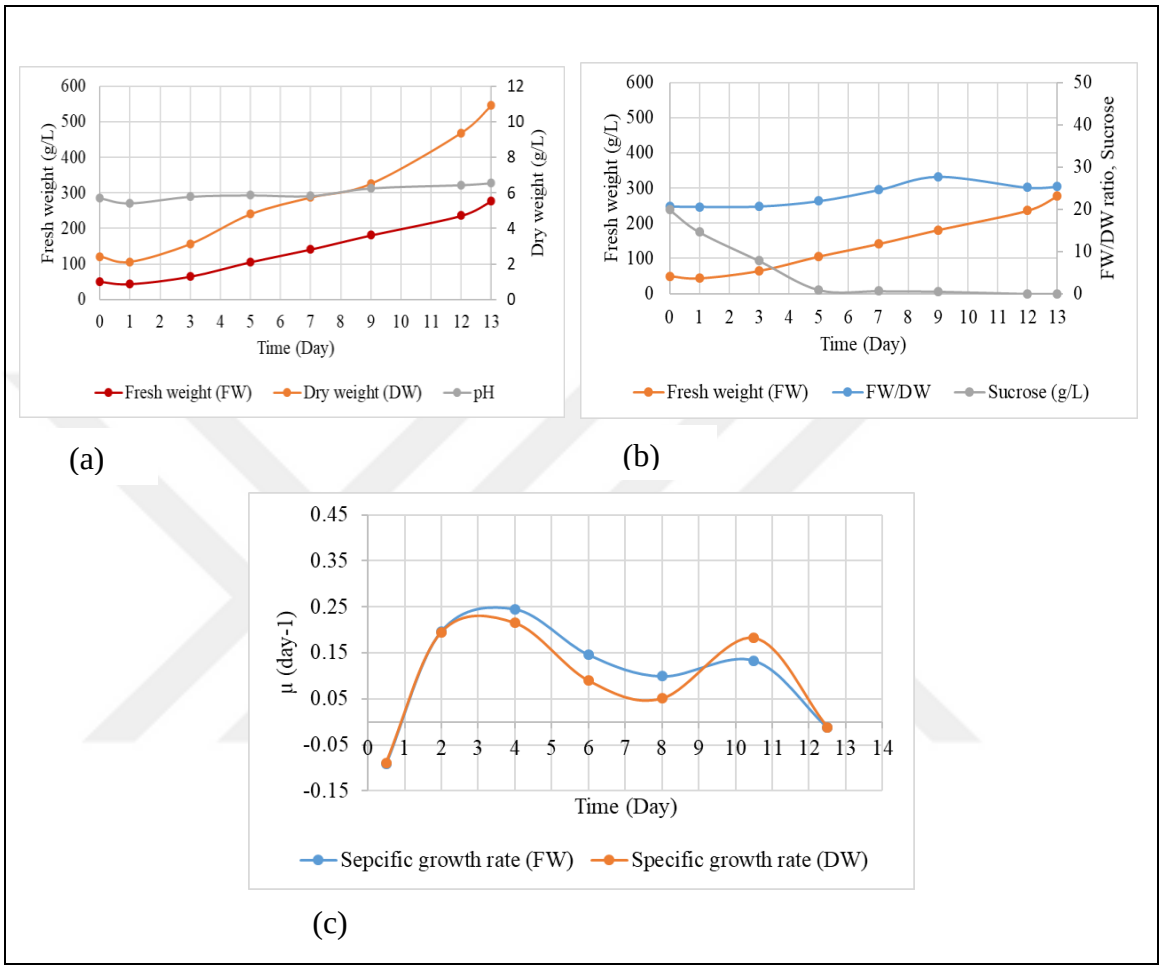


Figure 5.66 Growth profile of *V. vinifera* cv. Öküzgözü CSC. (a) FW (standard deviation was found between 0.01-0.1, DW (standard deviation was found between 0.004 - 0.1) and pH (standard deviation was found between 0.001-0.02), (b) ratio between FW/DW and sucrose consumption, and (c) Specific growth rate (μ)

5.15.1. Effect of sucrose concentration on cell biomass of *V. vinifera* cv. Öküzgözü cell suspension culture

To optimize the concentration, sucrose was used at 2% and 3% concentrations in *V. vinifera* cv. Öküzgözü CSC. The optimum ratio was found to be 3 % for biomass production in *Viti's vinifera* cv. Öküzgözü. Medium supplemented with 2% sucrose induced 276.9 g/L and 10.91 g/L maximum FW and DW cells at day 13, respectively. On the other hand, in the medium

with 3% sucrose, maximum FW and DW cells weight was obtained at day 13, reaching 304.2 g/L and 13.32 g/L, respectively (Figure 5.67 a,b).

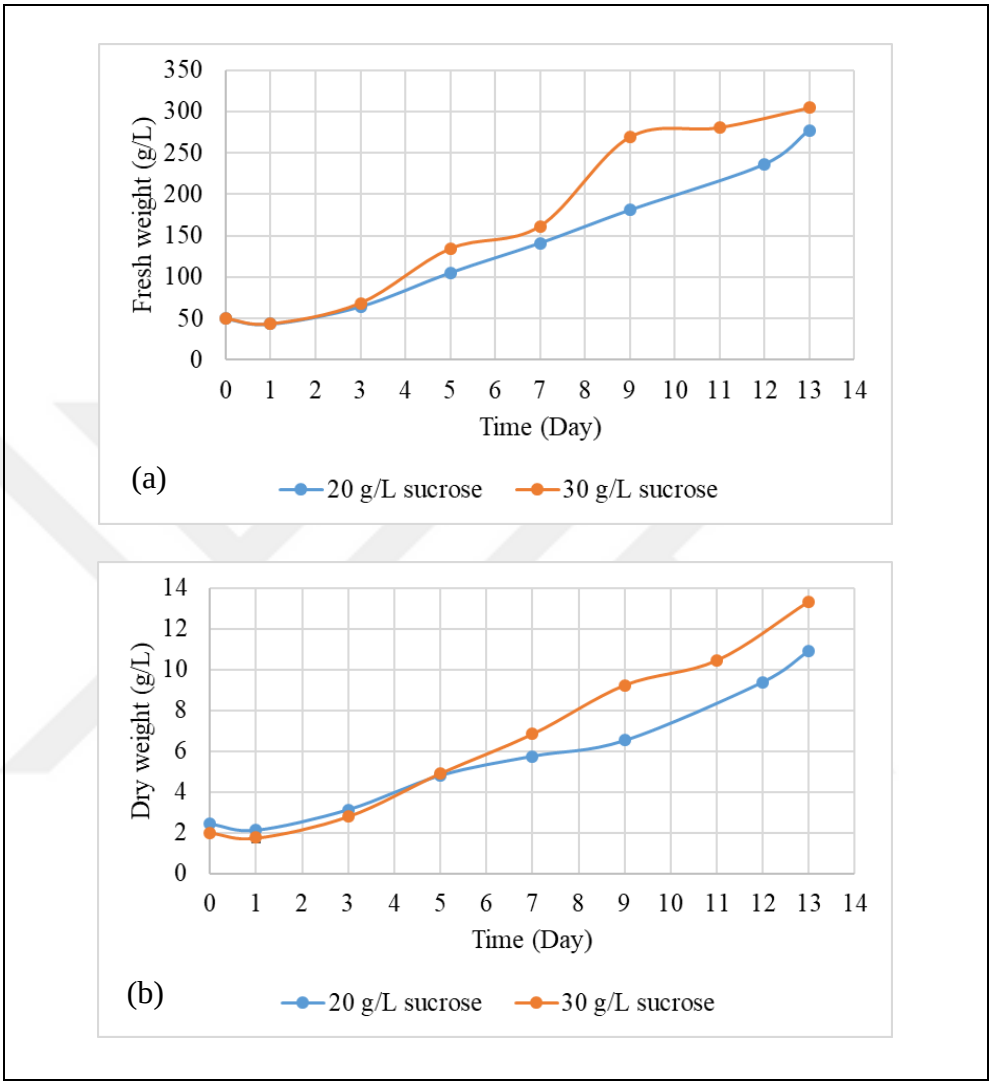


Figure 5.67. Effect of sucrose concentration on cell biomass of *V. vinifera* cv. Öküzgözü CSC. (a) FW, and (b) DW. FW (standard deviation was found between 0.01-0.19), DW (standard deviation was found between 0.01 - 0.22)

5.15.2. Effect of MeJA on cell biomass of *V. vinifera* cv. Öküzgözü cell suspension culture

Figure 5.68 a, b shows the FW and DW of *V. vinifera* cv. Öküzgözü cells treated with MeJA decreased based on the FW and DW reached in control cells. This indicated, after elicitation, some cells died so that cell growth decreased (133) (126). The effect of MeJA on cell growth

has been reported in many articles and this effect has been suggested to be caused by the diversion of the carbon allocation, favoring the activation of the secondary metabolism over the primary metabolism (133). Cell growth affected in elicitor-treated cells was showed also by cell viability measured by fluorescence microscopy of cells treated with FDA (Figure 5.70).

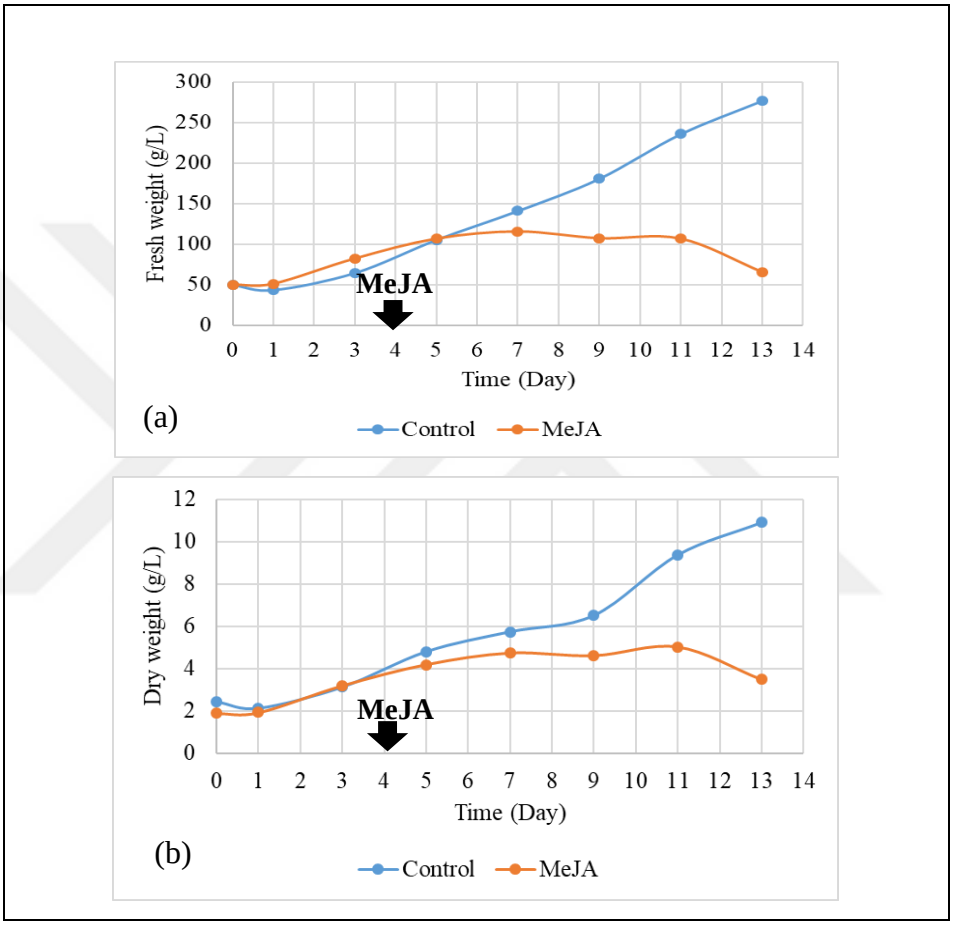


Figure 5.68. Growth profile of *V. vinifera* cv. Öküzgözü CSC before and after elicitation with MeJA. (a) FW, and (b) DW. After elicitation for FW (standard deviation was found between 0.001-0.23), and for DW (standard deviation was found between 0.02 - 0.18)

5.16. CELL DENSITY ANALYSIS OF *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

Between 2 g and 2.5 g as an initial density added on 50 ml media, the FW measured on day 7 showed a significant difference at level α=0,05 and no significant difference at α=0.01 (Figure 5.69 a).

Between 2 g and 3.1 g as an initial density added on 50 ml media, the FW measured on day 7 showed a significant difference at level α=0,05 (Figure 5.69 a).

Between 2.5 g and 3.1 g as an initial density added on 50 ml media, the FW measured on day 7 showed a significant difference at levels α=0,05 and α=0.01 (Figure 5.69 a).

The results of FW between 50 ml and 100 ml media volume showed significant differences at levels α=0,05 and α=0.01 (Figure 5.69 a,b).

Between the initial FW and final FW on day 7 was found a significant difference at levels α=0,05 and α=0.01 (Figure 5.69 a,b).

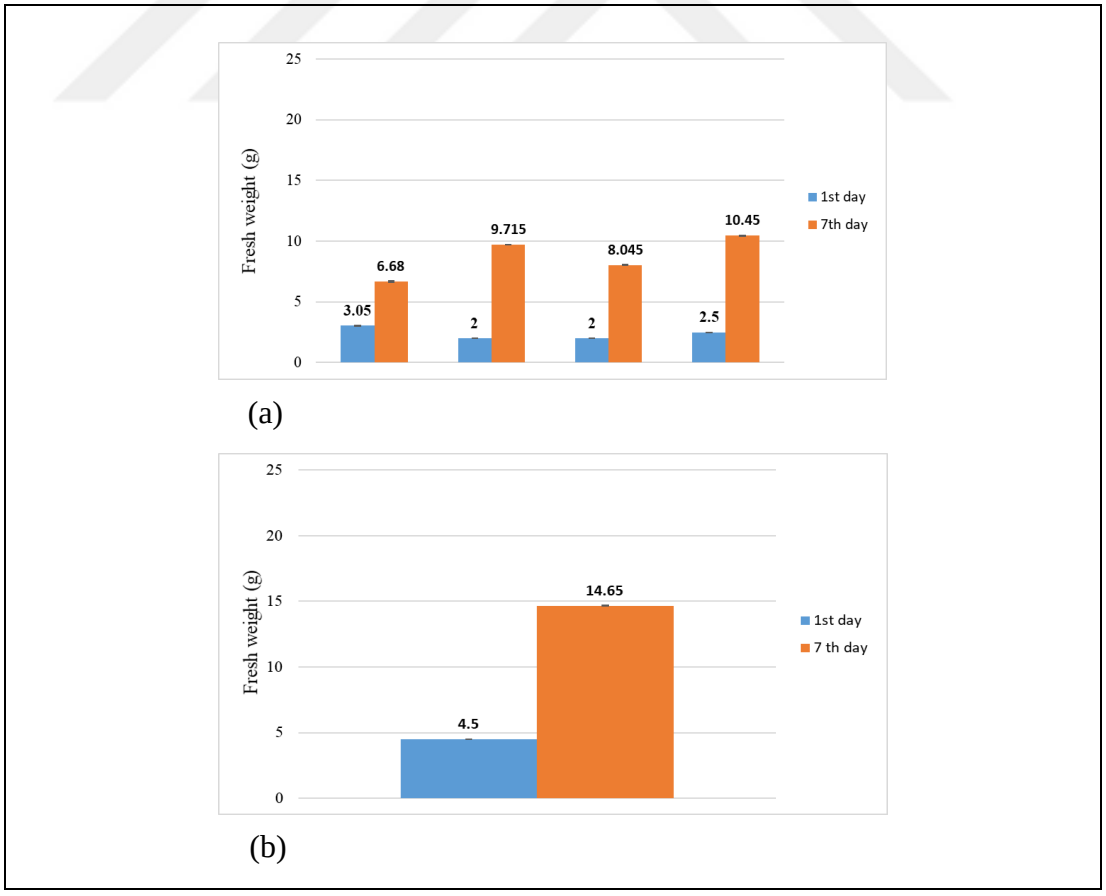


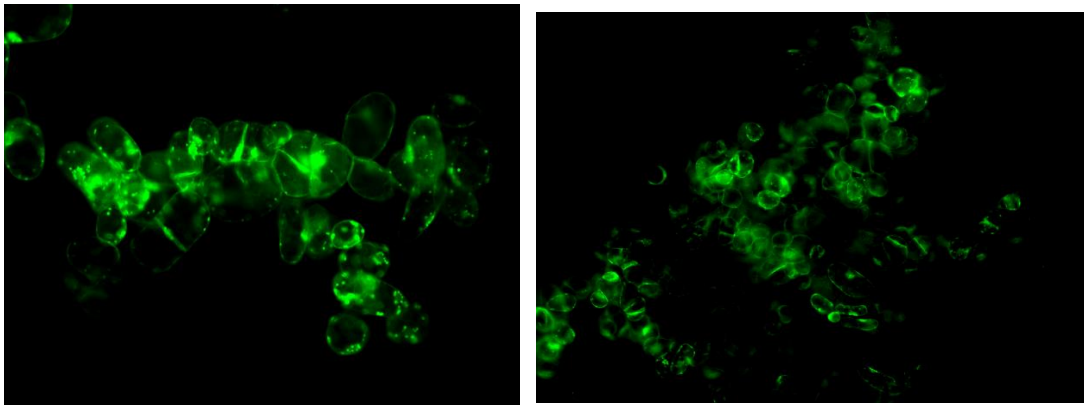
Figure 5.69. Cell density analysis in *V. vinifera* cv. Öküzgözü CSC. (a) 50 ml media/250 ml volumetric flask, and (b) 100 ml media/250 ml volumetric flask

5.16.1. Cell density analysis between *V. vinifera* cv. Öküzgözü and *Vitis labrusca* cv. Isabel cell suspension culture

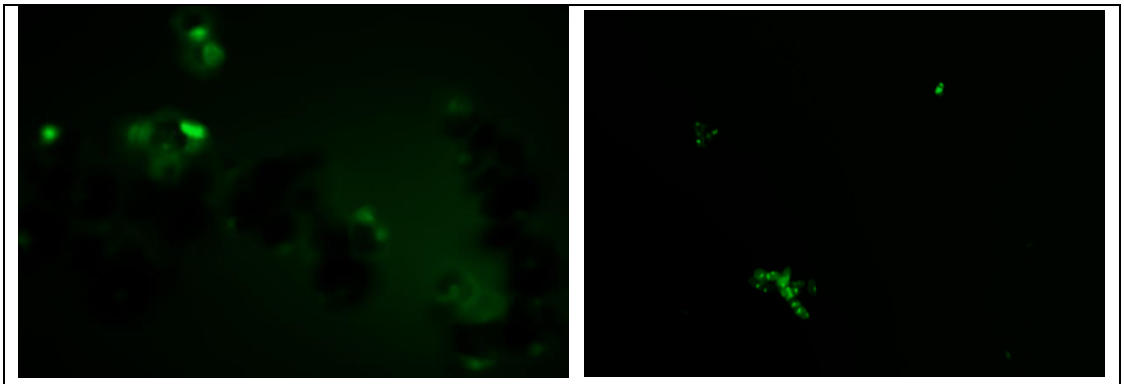
Between *V. labrusca* cv. Isabel and *V. vinifera* cv. Öküzgözü found a significant difference at level α=0,05 and α=0,01 on FW of day 7 when the initial density was 2 g.
Between *V. labrusca* cv. Isabel and *V. vinifera* cv. Öküzgözü found a significant difference at level α=0,05 on FW of day 7 when initial density was 2.5 g and 3g.
Between *V. labrusca* cv. Isabel and *V. vinifera* cv. Öküzgözü was not found a significant difference at level α=0,05 or α=0,01 on FW of day 7 when the initial density was 2,5 g and 4.5 g.

5.17. CELL VIABILITY TEST OF *V. VINIFERA* CV. ÖKÜZGÖZÜ CELLS WITH FLUORESCENCE MICROSCOPY

Cells treated with MeJA stopped growing. In comparison to control cells, the fluorescence microscopy measurement of cell viability in cells treated with FDA also appears to be impacted. (Figure 5.70 a,b).



(a)



(b)

Figure 5.70. Viability test of *V. vinifera* cv. Öküzgözü cells cultivated into VvSCIM13 media. (a) before elicitation, and (b) after elicitation

5.18. SUGAR ANALYSIS FOR *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

As shown in Figure 5.72a, in *V. Vinifera* cv. Öküzgözü CSC, sucrose was decomposed by the cell wall into fructose and glucose for 24 hours. Sucrose concentration at day 0 was 20 g/L (Figure 5.70) and was decreased up to 0.514 g/L on day 9 and after the 9^{th} day, no sucrose was found in the media. During sucrose hydrolysis, the growth of cells was slower compared to when it was completed and only glucose and fructose were present in the media. By consuming both fructose and glucose with maximum specific rates of 0.02/day and 0.3/day, respectively, *V. Vinifera* cv. Öküzgözü cells entered their exponential phase (Figure 5.72 b).

For elicited media of *V. Vinifera* cv. Öküzgözü CSC, the sucrose was decomposed into fructose and glucose by cell wall invertase for 24 hours and was depleted after day 7 (Figure 5.72c). Glucose and Fructose were consumed at maximum specific rates (0.17/day and 0.19/day respectively) (Figure 5.72 d). After MeJA was added to the media, only hexoses were left in the medium. In elicited media of *Viti's vinifera* cv. Öküzgözü CSC glucose and fructose were taken up at an equal specific rate as reported by (288) for rice CSC. Thus, the relative rates of transport of these sugars seem to differ between different plant species (Figure 5.72c).

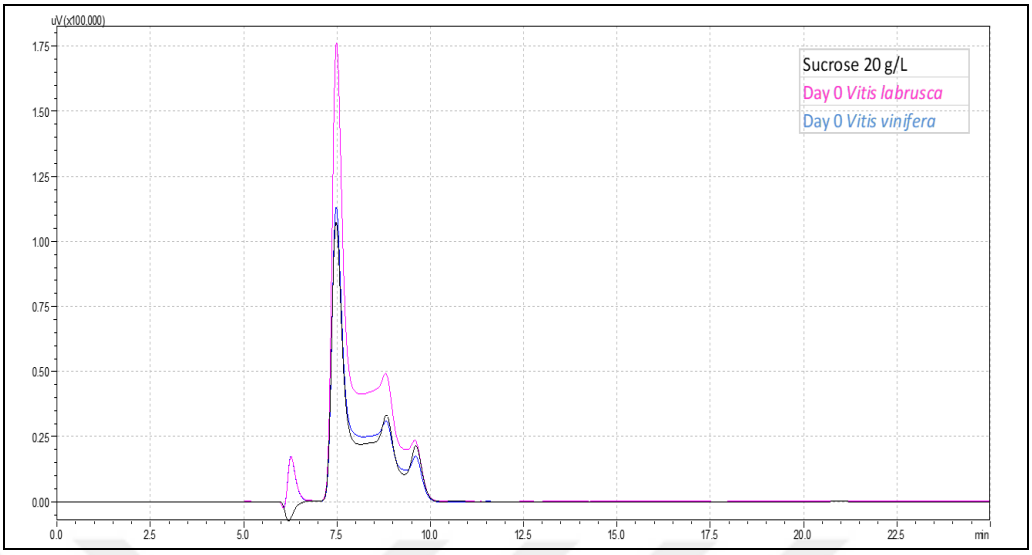


Figure 5.71. HPLC chromatogram of 20 g/L sucrose (standard) and the concentration of sucrose at day 0 for *V. vinifera* cv. Öküzgözü CSC (20 g/L)

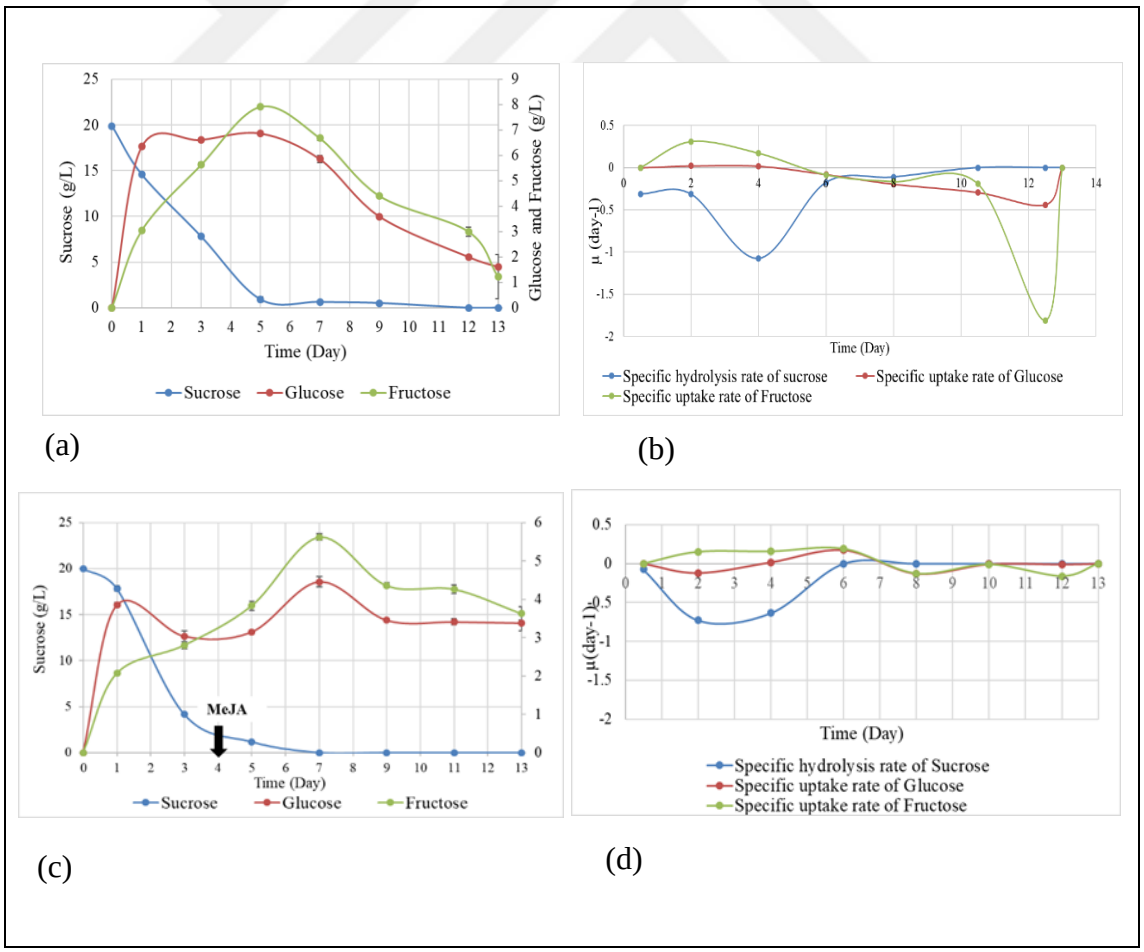


Figure 5.72. Sugar analysis and specific rate of sugar uptake in CSC of *V. Vinifera* cv. Öküzgözü. (a) (b) Control media, and (c) (d) Elicited media

5.19. EXTRACELLULAR TRANS-RESVERATROL PRODUCTION FROM *V. VINIFERA* CV. ÖKÜZGÖZÜ CELL SUSPENSION CULTURE

Extracellular t-resveratrol was identified and quantified by comparison with the authentic standard of 98 % purity resveratrol based on its retention time. Under these HPLC conditions, the retention time of resveratrol was 16.4922 min. The calibration curve was obtained from the increases in the peak areas of standard t-resveratrol which was observed at minutes 16.4922 min (Figure 5.73). The X-axis intercepts were taken into account for t-resveratrol amount determination. The calibration curve of standard t-resveratrol is shown in Figure 5.74.

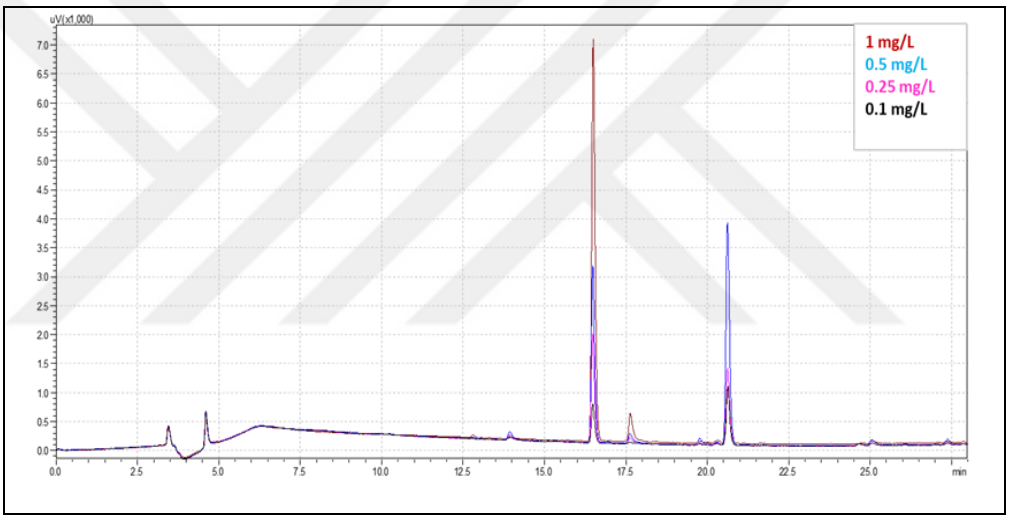


Figure 5.73. HPLC chromatogram of standard t-resveratrol for *V. Vinifera* cv. Öküzgözü

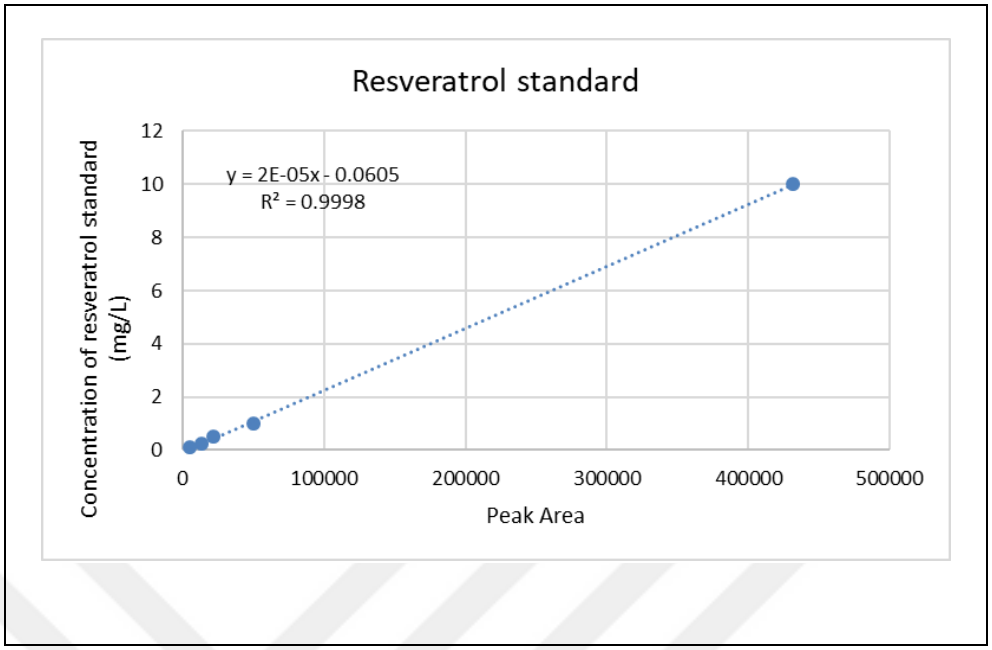


Figure 5.74. The calibration curve of standard t-resveratrol *V. Vinifera* cv. Öküzgözü

t-resveratrol production was monitored in *V. Vinifera* cv. Öküzgözü CSC before and after supplying MeJA on CSC. Figure 5.75 represent an HPLC chromatogram showing the peaks of extracellular t-resveratrol production in *V. Vinifera* cv. Öküzgözü.

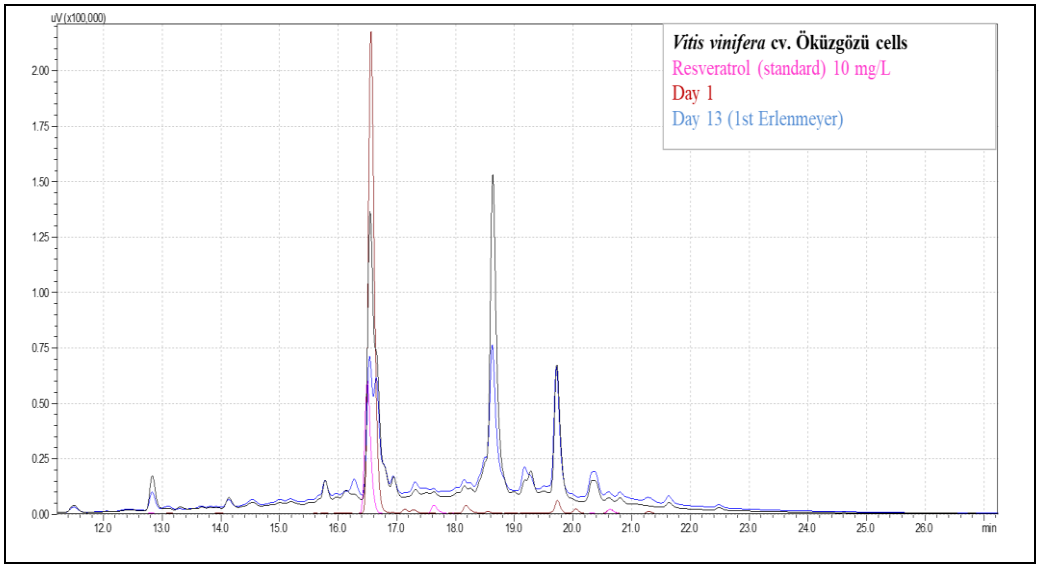


Figure 5.75. HPLC chromatograms of extracellular t-resveratrol from *V. Vinifera* cv. Öküzgözü CSC (Day1-before elicitation; Day 13-after elicitation with MeJA)

As shown in Figure 5.76 a, in the control group of *V. Vinifera* cv. Öküzgözü CSC, the highest amount of t-resveratrol was found in cell culture harvested on day 1 (84.8 mg/L) but it showed a sharp decrease on day 3, day 5, and day 7. After day 7, no resveratrol was found in control samples. In the *V. Vinifera* cv. Öküzgözü cell culture treated with MeJA (100 µM), t-resveratrol significantly increased after day 4 (when cells were treated with MeJA). On the 13th day, the level of t-resveratrol reached around 27, 34 mg/L. As shown in Figure 5.76 b, resveratrol stimulated its production and it prevented the growth of cells because its production is antagonistic with cell division metabolism. By its defensive role, t-resveratrol induced cell death in *V. vinifera* cv. Öküzgözü cells (258). In the literature was found also that t-resveratrol stimulated its production and it prevented the growth of cells which is under our results (258) (133) (126).

There are many articles found about using different varieties of *Vitis vinifera* in CSC for t-resveratrol production where t-resveratrol production was found in a lower amount than results showed in this thesis (referred to Table 2.4). One article was found about using the *V. vinifera* cv. Öküzgözü CSC for t-resveratrol production (177) where t-resveratrol production is in lower amount than in this thesis.

Differences in t-resveratrol levels among *V. labrusca* cv. Isabel and *V.vinifera* cv. Öküzgözü could be related to their different levels of defense response. It is critical to choose genotypes that can produce the maximum level of t-resveratrol because the *Vitis* species' response to elicitation in the presence of MeJA under similar conditions varied widely. Also, this difference in t-resveratrol might be on the level of sucrose in the culture medium (30 g/L sucrose for *V. labrusca* cv. Isabel CSC and 20 g/L sucrose for *Viti's vinifera* cv. Öküzgözü CSC as was studied by Navarro et al (133). The increase in carbon source might have a negative or positive effect on SM concentration, therefore this article studied the effect of different sucrose concentrations (1%, 2%, and 3%) on extracellular production of t-resveratrol in Monastrell CSC. Their results showed that the maximal amount of t-resveratrol production is dependent on both elicitors and sucrose concentration in the culture media. Thus, when grapevine SCC was elicited in a culture medium with 1% sucrose, the highest amount of t-resveratrol production (1,006.9 ± 58.1 µmol t-R /g DW) was reached using the lowest MeJA dose (25 µM). The highest amount of t-resveratrol production using 3% sucrose was obtained at 50 µM MeJA (1,210.8 ±40.1 µmol t-R/g DW). Conversely, the highest amount of t-resveratrol using 2% sucrose was reached at 100 µM MeJA (1,447.7 ± 60.4 µmol t-R /g DW) which is under our results for both species.

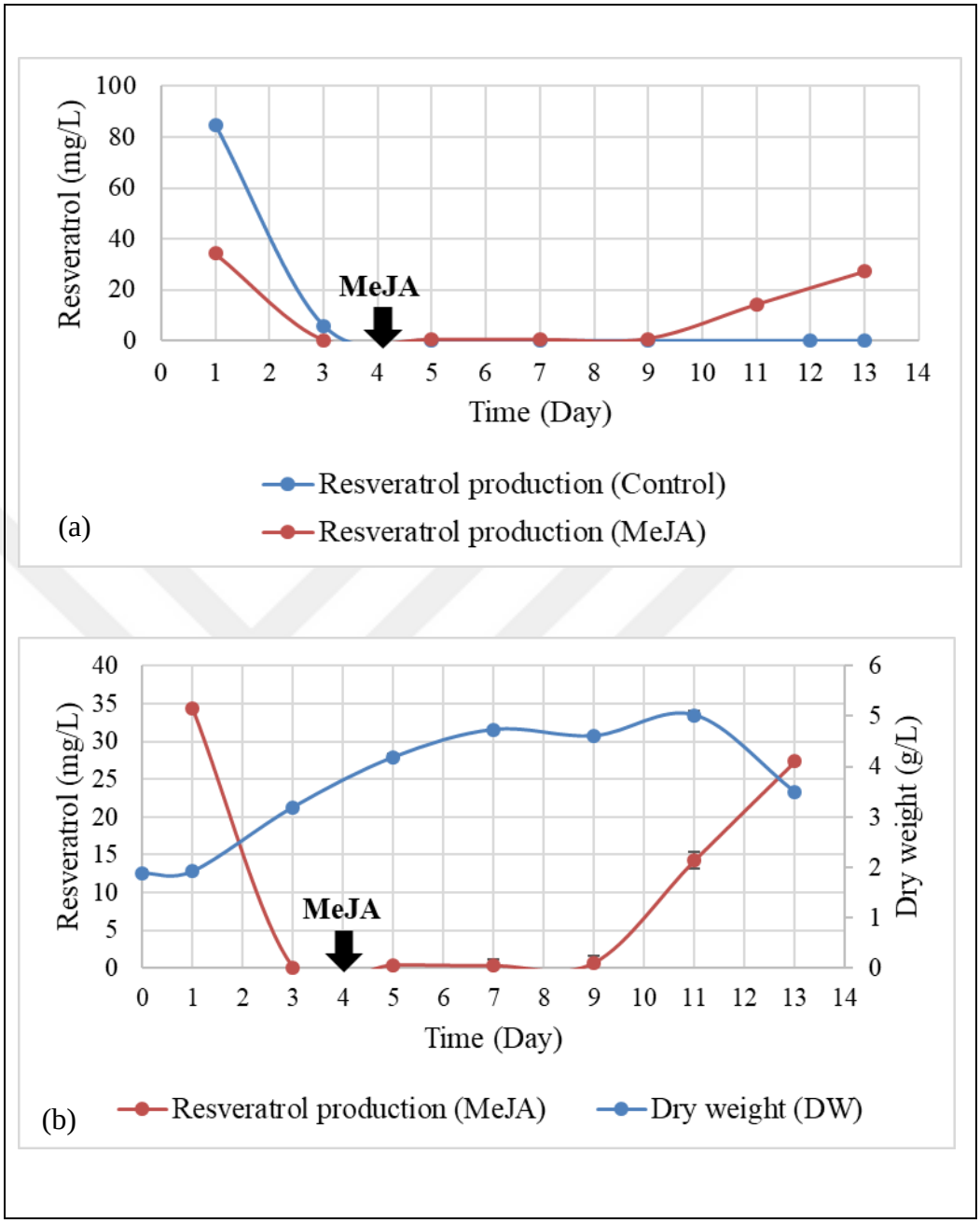


Figure 5.76. Extracellular t-resveratrol production in *V. Vinifera* cv. Öküzgözü CSC. (a) Control and elicited samples and (b) Correlation between t-resveratrol production and DW

6. CONCLUSION AND FUTURE PROSPECTS

The look for novel PSMs ought to be a priority in the present and future because most of the natural products that are required for food, medicine, and industry are of plant origin. Recently, the market for natural plant products has extended quickly, and this trend will continue since more people choose to utilize natural products. Future technological advancement in PCC will contribute more to the market.

The scale-up of PCSC in the bioreactor is required to develop any form of biotechnological process and the application of a bioreactor system for the cultivation of PCSC is an attraction because it holds great promise for the pharmaceutical and food industry. Many examples presented in this thesis, lead to consider the scale-up production of different SM with therapeutic value. However, it involves many challenges due to difficulties in scale-up, because of the plant cell features.

The usefulness of t-resveratrol is increasing due to its functions as anti-cancer effects, anti-aging effects, and cardiovascular diseases. It is important to highlight the t-resveratrol application in the pharmacy and cosmetic industries due to its biological activities and its current uses. t-resveratrol is produced by different plant species such as grapes, peanuts, and pine trees. Compared to all other natural sources, the grape has the highest concentration of t-resveratrol. t- Resveratrol can be found in the skin, leaf, stem, petiole, and root of grapes. Also, t-resveratrol was found in red wine by a few articles (289) (290) (291) (292). According to (292), the resveratrol content in red wine was found between 0.352-1.99 mg/L. Many factors affect the presence and content of t-resveratrol in wine such as environmental conditions, varieties of grapevines, vinification techniques, stress conditions, fermentation, and the post-fermentation process.

On the other hand, the use of *in vitro* culture such as PCSC is one suitable way for t-resveratrol production, due to the many advantages such as manipulation of environmental conditions, free of climatic changes or soil conditions, less differentiated cell populations, avoidance of harmful biological impacts (microorganisms and insects) that affect maintaining the production system, the ability to choose cultivars with higher plant cell production, automation control in cell development, and regulation of the metabolic process. Further, using PCSC, a large number of plantlets and better control for scale-up can be achieved. Because of not limited mass transfer is enabled to control the important process parameters including T, pO_2, pH, and substrate concentrations (130).

Using elicitors for the production of t-resveratrol should be considered a considerable route of its biosynthesis. Through elicitors, different varieties of *V. vinifera* (L.) might be improved for their production of t-resveratrol and its derivatives without using genetic engineering. Based on the research included in the thesis, different varieties of *Vitis* (L.) and different combinations of elicitors are used for enhancing t- resveratrol production.

We report here for the first time the data for biomass and t-resveratrol production by using *V. labrusca* cv. Isabel in Erlenmeyer and Stirred tank reactor (STR).

Successfully the protocol was modified and worked for the sterilization of *V. labrusca* cv. Isabel fruits where the immature fruits were washed under running water for 15 minutes, then surface-sterilized with 70% (v/v) EtOH for 7 minutes and rinsed twice with sterile dH_2O for 1-minute intervals. The fruits were then placed into 2 % (v/v) NaOCl for 15 minutes and rinsed with sterile dH_2O for 1-minute intervals 5-7 times.

The protocol successfully was modified and worked for sterilization of *V.Vinifera's* cv. Öküzgözü leaves and petioles where the leaf tissues were surface-sterilized with 2% NaOCl for 10 minutes and then were rinsed 5 times with sterile dH_2O whereas petioles were surfaced with 5% NaOCl for 10 minutes and then rinsed with sterile dH_2O 5 times.

Media compositions supplemented with 3.164 g/L Gamborg B5 medium including vitamins, 30 g/L sucrose, 250 mg/L CH, 215 µg/L kinetin, 93µg/L NAA, and 6.8 g/L plant agar were successfully modified and worked for callus induction from *V. labrusca* cv. Isabel fruits.

Media compositions supplemented with 3.164 g/L B5 media, including vitamins, 30 g/L sucrose, 250 mg/L CH, 0.1 mg/L NAA, and 0.2 mg/L kinetin were successfully modified and worked for suspension culture of *V. labrusca* cv. Isabel.

Media compositions supplemented with 3.164 g/L B5 media including vitamins, 20 g/L sucrose, 250 mg/L CH, 1 mg/L NAA, 0.2 mg/L kinetin, and 7 g/L plant agar were successfully modified and worked for callus induction of *V. Vinifera* cv. Öküzgözü.

Media compositions supplemented with 3.164 g/L B5 media including vitamins, 30 g/L sucrose, 1 mg/L NAA, and 0.2 mg/L kinetin were successfully modified and worked for suspension culture of *V. Vinifera* cv. Öküzgözü.

Protocol for extracellular extraction of t-resveratrol from *V. labrusca* cv. Isabel CSC and *V. Vinifera* cv. Öküzgözü CSC and protocol for intracellular extraction of t-resveratrol from *V. labrusca* cv. Isabel's CSC was modified and worked successfully.

MeJA elicitor at 100 µM concentration alone worked successfully for t-resveratrol production in both species. The FW and DW of treated cells with MeJA decreased (148 g/L

FW and 254 g/L FW; 6 g/L DW and 10 g/L DW) based on the FW and DW reached in control cells of *V. labrusca* cv. Isabel SCC (487 g/L FW and 514 g/L FW; 11.65 g/L DW and 14.57 g/ L DW). On the other hand, the pH profile shows an initial decrease during the lag phase (pH=5.63) and an increasing trend before adding the MeJA (pH=6.44). After MeJA was added, the pH started to decrease. In the second study performed in *V. labrusca* cv. Isabel SCC, the FW of treated cells with MeJA (100 µM) ß-CD (50 Mm), and a combination between MeJA and ß-CD (100 µM+ 50 Mm) decreased also based on the FW reached in control cells.

FW and DW of *V. vinifera* cv. Öküzgözü cells treated with MeJA decreased (106.62 g/L FW and 5 g/L DW) based on the weight reached in control cells (276.92 g/L FW and 10.91 g/L DW).

The biomass concentration reached in control cells of *V. labrusca* cv. Isabel (14.57 g/L DW) and *V. vinifera* cv. Öküzgözü (13.32 g/L DW) is higher than in other studies using different varieties of *V. vinifera* (L): 11 g DW/L (133), 10.9 g DW/L (124). A comparable result of biomass (14.57 g/L DW) was reached with another study that used another variety of *V. Labrusca* (*Vitis labruscana* (L.) cv. Campbell Early) (16 g/L DW) (267).

The highest amount of t-resveratrol in *V. labrusca* cv. Isabel CSC was found to be 6.78 mg/L (intracellular) and 28.23 mg/L (extracellular), whereas in *V. Vinifera* cv. Öküzgözü CSC it was found to be 84.8 mg/L (extracellular) via HPLC. Differences in t-resveratrol levels among *V. labrusca* cv. Isabel and *V. vinifera* cv. Öküzgözü could be related to their different levels of defense response. It is critical to choose genotypes that can produce the maximum level of t-resveratrol because the *Vitis* species' response to elicitation in the presence of MeJA under similar conditions varied widely.

There are many articles found about using different varieties of *V. vinifera* in CSC for t-resveratrol production where t-resveratrol production was found in a lower amount than results showed in this thesis (referred to Table 2.4). One article was found about using the *V. vinifera* cv. Öküzgözü CSC for t-resveratrol production (177) where t-resveratrol production is in lower amount than in this thesis also.

V. labrusca cv. Isabel cells were scaled up from shake-flasks to a 6 L STR under the controlled conditions of batch culture.

Setting the inoculum cell density at 45 g/L, setting the initial airflow to 0.06 L/min, using a PID control circuit between 0.06-0.8 L/min to avoid the dropping of dissolved oxygen concentration below a certain threshold, and using light resulted in the successful growth of

cells and t-resveratrol production in STR. The results presented here provide useful information about the use of light as a physical elicitor to increase the amount of t-resveratrol in *Vitis* plant CSC in a shake flask and STR.

Effect of CH in biomass and t-resveratrol production in *V. labrusca* (L.) cv. Isabel CSC in STR is *de novo* studied. Setting the mixing to 75 rpm, and the initial aeration rate to 0.06 L/min and with the presence of CH in media led to the highest biomass of 11.8 g DW/3L (5^{th} day) representing 4.52-fold higher biomass from the initial inoculation, whereas without CH in media, the highest biomass was revealed as 13.1 g DW/3L (8^{th} day) representing 5.02-fold increase. t-resveratrol production in STR with or without CH supplementation was found to be around 4 mg/L and 6 mg/L respectively. Besides, in response to light, accumulation of biomass and t-resveratrol were found to be 300 g FW /3L and 23.26 mg/L respectively, in STR. This study based on improvement in resveratrol production from *Vitis* CSC will shed light on future studies. The biomass concentrations reached in the bioreactor are comparable to other studies: 2.3 g DW/L (278) and 9.8 g/L (258) DW, 50 g/L FW (5^{th} day) (1) that is comparable with the fresh weight achieved in trial 4 (58 g/L FW), showing a good adaptation of the *V. labrusca* cv. Isabel CSC in bioreactor conditions.

V. vinifera cv. Öküzgözü callus was successfully cultivated in the media with CH, but *V. vinifera* cv. Öküzgözü CSC could not be established in presence of CH, which shows here for the first time the effect of CH in the suspension culture of *V. vinifera* cv. Öküzgözü.

Experiments conducted in a flask with CH in the media revealed the biomass increase to 14.57 g DW/L (11^{th} day), whereas without CH, 14.48 g DW/L (9^{th} day) biomass was obtained.

Despite the studies performed far away, further research is needed to improve the production of t-resveratrol. Since the preparation of t-resveratrol generally through plant extraction or chemical synthesis has limitations, therefore the use of bioreactors for scaling up the production of t-resveratrol presents a powerful route. Developing a model for the bioprocess, testing many physical parameters including temperature and pH, optimization of the parameters including the media composition, the aeration rates, and the speed of mixing, using different elicitors needed to be further investigated.

Furthermore, the right bioreactor with a low-shear impeller and the choice of the proper cultivation mode is necessary to increase the production of t-resveratrol. It has been shown, how significant is the optimization of culture and environmental conditions to increase the

productivity of t-resveratrol. Methods such as elicitor treatment, immobilization, and the use of two-stage due to non-related growth of t-resveratrol should be combined appropriately to improve the production of t-resveratrol.

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