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Ciências

Resveratrol production strategies: their influence on cell physiology and plasmid stability

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Resumo Alargado

A saúde e a alimentação são assuntos que suscitam grande interesse para a população em geral. O anti-envelhecimento e a proteção contra doenças são o objeto de inúmeras investigações, muitas delas envolvendo compostos naturais produzidos por plantas. As plantas são os mais antigos medicamentos do Homem e, atualmente, alguns dos compostos por elas produzidos são usados em várias indústrias, como a cosmética e a farmacêutica. Um dos compostos que tem suscitado bastante interesse devido às suas propriedades benéficas para a saúde é o resveratrol.

O resveratrol (3, 5, 4'–*trans*-hidroxistilbeno) é um polifenol e uma fitoalexina (metabolito secundário de plantas) pertencente à família dos estilbenos. Este composto pode ser encontrado em forma livre ou glicosilada – e ambas existem nas formas isoméricas *cis* e *trans*. *cis*-Resveratrol tem um máximo de absorção aos 286 nm, enquanto no isómero *trans* o máximo é atingido aos 306 nm. Esta fitoalexina não-flavonóide é produzida em plantas como resposta ao *stress* biótico (infeções por fungos ou bactérias) e abiótico, como radiação UV, calor ou lesão. Está presente em várias famílias de plantas e em pelo menos 72 espécies, mas encontra-se sobretudo nas uvas e amendoins. É encontrado frequentemente nos vinhos tintos, que pela ação antioxidante do resveratrol, são muitas vezes reconhecidos como cardioprotectores. O resveratrol reduz a morte celular e a sua atividade anti-inflamatória leva ao decréscimo das espécies reativas de oxigénio, sendo por isso eficaz no combate ao *stress* oxidativo, protegendo os componentes celulares. Este composto polifenólico demonstra ainda benefícios noutras áreas, agindo como agente antimicrobiano. O resveratrol possui também propriedades anticarcinogénicas; porém, a sua ação mais conhecida é provavelmente a ação anti-envelhecimento. Ainda há poucos dados sobre o metabolismo de resveratrol nos humanos, mas os últimos estudos em animais indicam que o resveratrol é facilmente absorvido após toma oral sobretudo no intestino delgado. No entanto, ainda é necessário mais informação sobre as doses e correlações com os efeitos.

Numa tentativa de criar formas alternativas à síntese química de resveratrol, alguns bioprocessos têm sido desenvolvidos para a sua produção em células de plantas e microrganismos recombinantes. Atualmente, o resveratrol pode ser produzido através de culturas de células vegetais, que têm a vantagem de serem totipotentes. Um dos sistemas de produção é a cultura de células suspensas, um sistema com boa reprodutibilidade e que não necessita de modificações genéticas. O máximo produzido por esta técnica foi 280 mg/L; porém, adicionando elicitores à cultura, pode atingir-se 5027 mg/L. Outra alternativa é a cultura de raízes e também a cultura de calos, ambas de rápido crescimento e bioquimicamente estáveis, mas com rendimentos relativamente baixos (1.5 mg/g DW e 33 mg/g, respetivamente). Desta forma, tendo como objectivo a produção de maiores quantidades, recorre-se aos microrganismos recombinantes, sendo os mais estudados a

bactéria *Escherichia coli* (*E. coli*) e a levedura *Saccharomyces cerevisiae* (*S. cerevisiae*). Tendo fácil manipulação e sendo largamente estudada, a bactéria *E. coli* foi o microrganismo que até à data produziu a maior quantidade de resveratrol de forma recombinante (1380 mg/L), enquanto a *S. cerevisiae*, que cresce rapidamente e é um organismo eucariota, produz menores quantidades de resveratrol: o seu máximo foi 391 mg/L. Desta forma, a *E. coli* é preferida como sistema recombinante para a produção de resveratrol. Embora o máximo produzido tenha sido atingido em culturas de células suspensas, os organismos recombinantes são geralmente preferidos devido à fácil manipulação e baixos custos associados.

Os microrganismos recombinantes *E. coli* e *S. cerevisiae* são cultivados, numa fase inicial, em balão, sendo importante a definição das condições de cultura como a temperatura, pH e agitação, assim como a composição dos meio de cultura, que deve ir de encontro aos requisitos nutricionais dos microrganismos em questão. No entanto, para obter um maior rendimento e controlo, este processo pode ser efetuado em bioreator. Por vezes, numa tentativa de otimizar as condições de cultura, testam-se vários fatores, um a um, até definir quais os pontos que mais favorecem a produção. Esta abordagem pode posteriormente ser aproveitada para fazer um desenho experimental (como o *Central Composite Rotatable Design*), no qual se conjugam vários fatores para determinar quais são os mais relevantes e influentes na produção do composto de interesse. A resposta do modelo gerado é submetida a uma análise estatística, obtendo-se uma resposta de superfície, que quantifica a relação entre os fatores a testar e as respostas obtidas.

Muitas vezes são usados métodos de monitorização paralelamente aos ensaios de produção. Neste caso, para avaliar a viabilidade celular e a instabilidade segregacional dos plasmídeos é usada a citometria de fluxo e real-time qPCR, respetivamente. Estes dois parâmetros são muito importantes no processo, uma vez que baixo crescimento celular ou células metabolicamente pouco ativas, assim como uma partição incorreta de plasmídeos para as células filhas podem levar a uma grande diminuição na produtividade final. A citometria de fluxo fornece em tempo real informação sobre a viabilidade celular, atividade enzimática ou conteúdo em ácidos nucleicos. É muitas vezes necessário recorrer a fluoróforos, como o BOX bis-(1,3-ácidodibutilbarbitúrico)trimetina oxonol, que avalia o potencial de membrana (se está ou não despolarizada), ou o iodeto de propídeo (PI), que avalia a permeabilidade da membrana. Estes dois compostos foram usados neste estudo para averiguar, através de citometria de fluxo, a viabilidade celular de fermentações em bioreator. Outro ponto que é necessário avaliar é a estabilidade segregacional dos plasmídeos, que é verificada usando real-time qPCR através da monitorização do número de cópias de plasmídeo (PCN). Esta técnica permite uma rápida quantificação de qualquer sequência alvo num curto espaço de tempo. Desta forma, avaliando-se parâmetros adicionais à fermentação, pretende-se maximizar a produção de resveratrol.

Devido ao elevado número de aplicações e também à elevada procura das indústrias por este composto, o objetivo geral deste estudo é produzir resveratrol em bioreatores usando microrganismos recombinantes (*E. coli* e *S. cerevisiae*), ao mesmo tempo que se monitoriza o processo para verificar a fisiologia celular (através de citometria de fluxo) e a instabilidade segregacional, medindo o número de cópias de plasmídeo através de real-time qPCR.

Embora a levedura *S. cerevisiae* não tenha sofrido transformação, a bactéria *E. coli* BW27784 foi transformada com os plasmídeos pAC-4CL1 e pUC-STS. Para analisar as quantidades de composto produzidas em fermentações, são geralmente usados métodos cromatográficos (cromatografia líquida ou gasosa) após as amostras terem sido submetidas a extração. De forma a quantificar o resveratrol nas amostras de meio de cultura, o método cromatográfico foi validado segundo regras internacionais. A extração de resveratrol foi efetuada usando uma extração líquido-líquido com acetato de etilo, sendo as amostras posteriormente injetadas num aparelho de cromatografia líquida de altíssima performance (HPLC) acoplado a um detetor de *diode array* (DAD). Foi usada uma coluna Zorbax C-18 a 25 ºC, tendo como fase móvel água, acetonitrilo e ácido acético nas proporções 66:33:0.1, a pH 3.4 e a um caudal de 1 mL/min. A curva de calibração foi feita com 7 pontos entre a concentração de 0.1 a 10 µg/mL, tendo-se obtido uma correlação superior a 0.99 usando uma ponderação estatística de $\frac{1}{x^2}$. A eficiência de extração foi de aproximadamente 100 % para os valores 0.1 e 10 µg/mL e o máximo desvio padrão obtido em todas as análises foi ± 0.41. Concluiu-se deste modo que, como os valores se encontravam dentro dos parâmetros definidos internacionalmente, o método é reprodutível, preciso e rigoroso, devido à pequena variação entre os valores analisados e obtidos. O método também é reprodutível devido ao baixo erro associado à precisão intradia (cujo valor máximo de desvio padrão foi de ± 0.22, podendo ser utilizado para a quantificação de resveratrol neste tipo de amostras.

De seguida, foram efetuados ensaios de *screening* para avaliar qual o microrganismo recombinante que permitia a obtenção de maiores níveis de produção de resveratrol. Para estes ensaios foram usados os dois microrganismos recombinantes em estudo, tendo sido avaliados vários parâmetros que poderiam influenciar a produção de resveratrol: temperatura, composição do meio, densidade ótica (OD_{600}) na altura da indução, concentração de indutor (ácido *p*-cumárico), agitação e, para a bactéria, foi também avaliada a influência do pH. No ensaio das concentrações de indutor, foram avaliadas 6 concentrações: 0, 1 ou 2, 5, 10, 15 e 20 mM. A produção máxima de resveratrol em *E. coli* foi atingida com 10 Mm de indutor, obtendo-se 105.65 µg/mL de resveratrol; enquanto 5 mM foi a concentração de indutor que levou a uma maior produção em *S. cerevisiae*; no entanto, devido ao caráter tóxico do solvente usado para preparar o indutor (DMSO), tanto a *E. coli* como a *S. cerevisiae* deixaram praticamente de crescer e produzir acima de 10 Mm. Quanto à densidade ótica na altura da indução, verificou-se que, em *E. coli,* a indução a densidades óticas inferiores a 0.2 tinha um impacto negativo na produção. Na levedura, este ensaio não demonstrou ter impacto na produção, embora o melhor resultado se tenha obtido a uma densidade de 1. No

estudo da influência da temperatura na produção de resveratrol, foram feitos ensaios a 25 ºC, 30 ºC, 37 ºC e 42 ºC, tendo sido verificado que a temperatura que permite a obtenção de concentrações mais elevadas de resveratrol é 30 ºC para ambos os microrganismos, onde se atingiu a produção máxima (83.10 µg/mL para a *E. coli* e 1.23 µg/mL para a *S. cerevisiae*). A influência da agitação na produção de resveratrol também foi avaliada (150, 200, 250 e 300 rpm) e concluiu-se que a agitação ótima para os dois microrganismos é diferente, sendo que para a *E. coli* é 250 rpm e para a *S. cerevisiae* é 200 rpm. No entanto, a agitação é um fator sem grande impacto na produção final, pois não existe grande variação nos valores da produção tendo em conta a mudança na agitação. Nos ensaios com diferentes composições do meio de cultura, foi testada a influência do tipo de fontes de carbono e azoto, tendo-se verificado que ambos os microrganismos utilizaram as maiores quantidades de nutrientes em detrimento da produção de resveratrol, que apesar das elevadas densidades óticas atingidas, apenas ficou próxima aos 100 µg/mL. Como nos ensaios anteriores a *S. cerevisiae* produziu sempre menores concentrações de resveratrol que a *E. coli*, esta foi seleccionada para os ensaios subsequentes. Desta forma, os estudos sobre o pH incidiram apenas neste microrganismo, e no ensaio realizado para o estudo da influência do pH na produção de resveratrol, verificou-se que o ponto óptimo de produção foi atingido a pH 7.

Após realização dos ensaios preliminares, os resultados foram usados para criar uma grelha de ensaios de *design* experimental (*Design of Experiments*, DoE). Uma vez que nem todos os fatores testados influenciaram de igual forma a produção de resveratrol em balão, para o desenho experimental foram apenas considerados os fatores temperatura, pH, concentração de indutor e a densidade ótica na altura da indução. Apesar de, estatisticamente, o modelo não ter sido validado, foi possível retirar conclusões acerca da produção de resveratrol nesta estirpe recombinante de *E. coli*. O máximo de produção obtido foi de 159.96 µg/mL, a maior concentração descrita até ao momento para esta estirpe. As condições para obtenção deste valor foram 4 mM de ácido *p*-cumárico adicionado à densidade ótica de 0.8, sendo o pH de 6.5 e a temperatura de 28 ºC. Este estudo revelou também que, embora haja fatores que influenciam grandemente a produção de resveratrol e crescimento bacteriano (como a temperatura e pH), verificou-se que a influência e interação com outros fatores têm um peso importante no resultado final, pois obtiveram-se concentrações elevadas de resveratrol quando alguns dos fatores do ensaio estavam mais distantes do ideal. Quanto aos resultados de fisiologia celular, pode concluir-se que cerca de 26 % das células se encontravam despolarizadas e 4 % estavam mortas no final de 30 horas de fermentação. Os elevados valores de células despolarizadas podem ser devido à falta de nutrientes no meio, que levou à quebra das funções básicas de manutenção nas células, como a manutenção do potencial de membrana. No entanto, elevadas concentrações de DMSO no indutor mostraram ter um efeito adverso na viabilidade celular. Quanto à análise da instabilidade segregacional, pode concluir-se que o PCN aumenta em ambos os plasmídeos das 22 às 30 horas, como se pode verificar no ensaio 8, em que os valores para as 22 e 30 horas oscilaram entre os 215 e os

1541 para o pAC-4CL e os 72 e 89 para o pUC-STS. Esta pode ser uma explicação para a produção mais elevada de resveratrol obtida às 30 horas de fermentação. Concluiu-se também que o plasmídeo pUC-STS é mais instável, pois as temperaturas usadas não favoreceram a sua indução, além de que contém um gene de resistência à ampicilina que favorece o aumento da instabilidade segregacional. No geral, os valores de PCN são mais baixos relativamente a outros estudos devido à carga metabólica imposta pelos plasmídeos, o que resulta em menores taxas de crescimento e aumenta a instabilidade segregacional.

Em suma, neste estudo foi possível a produção de elevadas quantidades de resveratrol em bioreator a partir de microrganismos recombinantes e recorrendo a ferramentas adequadas de monitorização. Este estudo pode ser um possível ponto de partida para a produção industrial deste composto e uma alternativa viável em relação à síntese química e ao consumo dos recursos naturais.

Palavras-chave

Resveratrol, Bioreator, HPLC-DAD, DoE, Citometria de Fluxo, Real-time qPCR.

Abstract

Resveratrol (3, 5, 4'-*trans*-hydroxystilbene) has been used since immemorial times in traditional medicine as an antimicrobial and antioxidant compound. Recent discoveries demonstrated other health benefits for this phytoalexin, such as anticancer and anti-ageing activities, making it desirable for the pharmaceutical, nutraceutical and cosmetic industries. This polyphenolic is a plant secondary metabolite mainly produced by peanuts and grapevines. Although plant cells were traditionally used as a biological alternative for resveratrol production, in recent years, recombinant microorganisms, such as yeast and bacteria, were proposed to improve resveratrol production. The present work describes resveratrol production in two recombinant microorganisms – *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) – its optimization of production in bioreactor using Design of Experiments (DoE) and the impact on cell physiology and plasmid stability, which was assessed by flow cytometry and real-time qPCR, respectively. For resveratrol quantification, a liquid-liquid extraction from culture media was performed using ethyl acetate and then a method for quantification in High Performance Liquid Chromatography – Diode Array Detector (HPLC-DAD) was validated. In order to assess which recombinant microorganism yielded higher resveratrol production, the influence of medium composition, pH, temperature, agitation, precursor concentration and optical density (OD_{600}) at the addition of precursor were evaluated for resveratrol production in shake flask using *E. coli* and *S. cerevisiae*. The data obtained were used to create a DoE approach in order to optimize resveratrol production in bioreactor. The bioprocess was monitored using the HPLC-DAD method for resveratrol quantification, flow cytometry to assess cellular viability and real-time qPCR to evaluate plasmid segregational instability. Shake flasks screening assays revealed a 30 times higher resveratrol yield by *E. coli* (about 100 µg/mL) when compared to *S. cerevisiae* (3.17 µg/mL), which led to the choice of the first microorganism for the scale-up optimization studies. Only the factors that had the highest impact on resveratrol production were considered for the DoE approach: temperature, pH, precursor concentration and optical density (OD_{600}) at the addition of precursor. A Central Composite Design, rotatable and full fractioned was used, which allowed the obtention of 159.96 µg/mL of resveratrol. The population of depolarized cells varied according to the conditions used, which sometimes resulted in a 10 % difference between higher and lower production assays. Plasmid segregational instability had also been observed and variations in the values of plasmid copy number (PCN) were noticed between 22 and 30 hours of fermentation, with the highest PCN values obtained at 30 hours, when also the highest amounts of resveratrol were obtained. It is possible to conclude that cellular viability and plasmid segregational instability affect significantly resveratrol production. In sum, this work outlines the optimization of resveratrol production in bioreactors using flow cytometry and real-time qPCR for bioprocess monitoring. It was demonstrated that using the appropriate tools to optimize and monitor resveratrol

production process, solutions can be found for mass production of this compound, providing an effective alternative to chemical synthesis and avoiding the depletion of natural sources.

Keywords

Resveratrol, Bioreactor, HPLC-DAD, DoE, Flow Cytometry, Real-time qPCR.

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List of Abbreviations

3-O-GT	3-O-glucosyltransferase
4CL	4-coumaroyl CoA ligase
BOX	Bis-(1,3-dibutylbarbituric acid)trimethine oxonol
C4H	Cinnamate 4 hydroxylase
CD	Cyclodextrins
CoA	Coenzyme A
CPR	Cytochrome P450 reductase
CV	Coefficient of variation
DAD	Diode Array Detector
DIMED	2,6-di-O-methyl-ß-cyclodextrin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DoE	Design of Experiments
DW	Dry weight
EDTA	Ethylenediamintetraacetic acid
FC	Flow cytometry
FDA	Food and Drug Administration
FSC	Forward scatter
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonisation
LDL	Low-density lipoproteins
LLE	Liquid-liquid extraction
MeJa	Methyljasmonate
mRNA	Messenger ribonucleic acid
OD_{600}	Optical density
PAL	Phenylalanine ammonia lyase
PBS	Phosphate-buffered saline
PCN	Plasmid copy number
pDNA	Plasmid deoxyribonucleic acid
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
R^2	R-squared
RAMED	Randomly Methylated-ß-cyclodextrin
RE	Relative error
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSM	Response surface methodology

SD	Standard deviation
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SSC	Side scatter
STS	Stilbene synthase
T-DNA	Transfer DNA
TAE	Tris-Acetate-Ethylene diamine
TAL	Tyrosine ammonia lyase
UV	Ultra-violet

Chapter 1

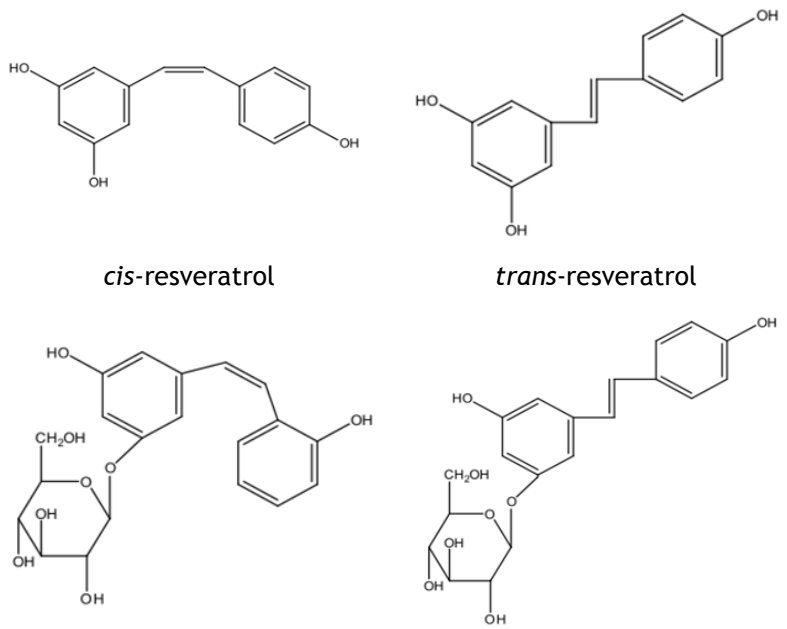
1. Introduction

In modern life, food and health are subjects of concern to society. In this perspective, the search for improved wellness, protection against disease and ageing are the starting point of several investigations and investments throughout the world. Current lifestyle leads to changes in nutrition, activity and obesity, which are linked with some chronic diseases [1]. Research has shown that a diet based on plant foods and less in dairy products and animal food is associated with lower rates of chronic disease and higher adult life expectancy [2]. Furthermore, plants are used since ancient times in a wide range of applications, from a healing source to a poison, from tonic to soap plants and cosmetics, dental care to fragrances and repellents [3].The use of plants remained over time and, nowadays, the global market for plant-derived products exceeds several billion dollars per year [4]. Plant compounds, such as polyphenols and phytoalexins, which include resveratrol, are widely investigated due to their broad range of biological activities, such as antimicrobial, anti-oxidant and anti-ageing [5-7].

1.1. Resveratrol

Resveratrol (3, 5, 4'-*trans*-hydroxystilbene) is a phytoalexin [8] – a low-molecular weight secondary metabolite with antimicrobial activity – and a polyphenolic compound that belongs to the stilbene family [9]. Chemically, resveratrol ($C_{14}H_{12}O_3$) is a white powder with a slight yellow cast, a molecular weight of 228 g/mol and a melting point of 253 - 255 ºC [10].

Resveratrol can be found in free (aglycone) or glycosylated form (piceid) and its oxidative dimerization leads to the formation of its polymer, the viniferins [10]. Both aglycone and piceid exists in *cis*- or *trans*- isomeric forms (figure 1) [8], because their two phenol rings (linked by a styrene double bond) generate the more stable form, *trans*-resveratrol; but, by UV photoisomerization, *trans*-resveratrol is converted to *cis*-resveratrol [11]. *cis*-Resveratrol has a maximum of absorbance at 286 nm, whereas the maximum absorbance for isomer *trans* is achieved at 306 nm. Resveratrol (both *cis* or *trans* isomers) are extremely light sensitive and when protected from light, *trans*-resveratrol is stable for at least 28 days in buffers with pH ranging from 1 to 7, while *cis*-resveratrol is degraded at pH 10.0 [12].



cis-resveratrol *trans*-resveratrol

cis-piceid *trans*-piceid

Figure 1. Structures of resveratrol derivatives and isomers [8].

Resveratrol-containing plants, such as Itadori plant (*Polygonum cuspidatum* syn. *Fallopia japonica*) were used in China and Japan for centuries as herbal folk remedies to treat various ailments, including bacterial, fungal and inflammatory illnesses, lipid atherosclerosis [12], heart disease and stroke [13]. Resveratrol was first isolated from white hellebore (*Veratrum grandiflorum O. Loes*) by M. J. Takaoka in 1940 [9], later in 1963 it was found in Itadori plant [10] and *trans*-resveratrol was discovered in *Vitis vinifera* (grape vine) in 1976 by P. Langcake and R. J. Pryce [12].

This non-flavonoid phytoalexin is produced by plants in response to biotic and abiotic stress. Biotic stress includes mainly microbial or fungal infections [14], but resveratrol also acts as a defense in case of nematodes or herbivores attack [6]. Abiotic stresses may result from mechanical injury [13], UV and far-infrared radiation [14], ultrasound, ozone and heat [10]. Resveratrol production by plants is also affected by their treatment with chemicals, such as cupric acid, salicylic acid, jasmonic acid, ethylene, aluminum chloride and aluminum sulfate [10]. Being a stilbene, resveratrol is a plant-specific natural compound produced by various plant families, including *Vitaceae*, *Dipterocapaceae*, *Gnetaceae*, *Pinaceae*, *Poaceae*, *Fabaceae*, *Leguminoseae* and *Cyperaceae* [6] and at least in 72 plant species [15]. Thereby, resveratrol can be found in grapes and grape products (particularly red wine), peanuts, several types of berries (cranberries, bushberries, blueberries and strawberries) [5], ferns [16], pines, legumes [13], white hellebore [6], pistachios [17], but also in flowers and leaves (such as eucalyptus, spruce, butterfly orchid and rheum) [15].

Resveratrol is present in higher quantities in peanuts and grapes, being commonly found in red wines [16], since it is a component of the grape's skin (up to 0.1 %) [18], but is primarily found in the glycosylated form [8]. Moderate consumption of red wine during meals is one of the main rules of Mediterranean diet [2] and has been proposed as explanation of the low incidence of heart disease in people with a high fat content diet [19]. This was noticed long ago, but in 1991, a television program demonstrated this paradox in the French population, being known as 'French Paradox' [20]. Resveratrol can act as antioxidant and an inhibitor of platelet aggregation [19] and, since it is similar to the synthetic estrogen diethylstilbestrol – known by cardioprotective benefits – meaning that the intake of this compound in red wine can provide an explanation for the 'French Paradox' [19]. Beyond this cardioprotective effect, resveratrol is also known by its action in many other areas and by its various benefits.

1.2. Resveratrol biological activities

After many tests to phytoalexins, there are numerous evidences for the clinical usefulness of resveratrol [14] – due to its wide range of biological activities – and, therefore, various mechanisms of action and targets [5]. Between the broad applications of resveratrol, the major biological activities are antimicrobial and antioxidant activities, cardio and neuroprotective, cancer chemoprotective, prevention of ageing, reduction of obesity and inflammation, among other health beneficial effects [5] (Table 1).

Table 1. Biological activities and health benefits of resveratrol.

Properties	Examples of benefits	References
Anti-ageing	Activator of sirtuin activity, repairing DNA, slowing senescence and fat mobilization.	[7, 14, 21]
Anticancer	Cytostatic or cytotoxic agent; Prevention of exponential growth of cancer cells; Inhibition of preneoplastic lesions.	[6, 14, 22]
Anti-inflammatory	Reduction of inflammatory cell damage; Decrease of reactive oxygen species (ROS).	[7, 22]
Antimicrobial	Inhibition of growth of human pathogenic bacteria and fungi.	[5, 23]
Antioxidant	Free radical scavenger; Protection low-density lipoproteins (LDL) against peroxidation; Use as food preservative.	[6, 22, 24]
Cardioprotective	Protection against acute ischemic stroke; Attenuation of oxidative stress after ischemia reperfusion.	[25]
Neuroprotective	Breaks down beta-amyloid aggregates; Up-regulates kinases in learning and memory centers of brain.	[26]

1.2.1. Antimicrobial activity

Antibacterial therapy is a powerful tool in the current clinical practice. However, microorganisms can adapt their defenses against the antibiotics used, developing resistance to several antibiotics, thus causing a public health problem [5]. Nowadays, there is a growing interest on antimicrobial compounds from natural sources, as resveratrol, due to consumers concern about their health. In fact, it has been shown that this compound has the ability to inhibit the growth of some human pathogenic bacteria, such as *Klebsiella pneumonia, Helicobacter pylori, Listeria monocytogens* and *Staphylococcus aureus* [5]. Resveratrol also has antimicrobial activity against fungi, such as *Candida albicans, Trichosporon beigeli* [5], *Epidermophyton floccosum* [23] and *Botrytis cinerea*, a common grapevine pathogen, slowing down or inhibiting the spread of this fungal infection [24].

1.2.2. Antioxidant

Several evidences showed that resveratrol acts both as a free radical scavenger and a potent antioxidant, owing to its ability to promote the activity of numerous antioxidative enzymes [6]. It is thought that resveratrol protects low-density lipoproteins (LDL) against peroxidation, leading to a regulated catabolism of LDL particles [22] and thus avoiding atherosclerosis [6]. Resveratrol also protects cellular and subcellular components, protecting cell membranes and preventing the effects of oxidative stress, leading to a reduction in cell death [22]. Being a strong antioxidant, resveratrol may be applied in the food industry as a preservative, preventing the oxidation of oils and margarine and suppressing food deterioration by molds due to its antifungal activity [24].

1.2.3. Anticancer properties

Resveratrol induces a complex array of effects on cells. This molecule is considered an antiproliferative agent for cancer, exerting its activity as a cytostatic or cytotoxic agent in cancer cells [6], inducing a complex array of effects that range from the inhibition of growth (preventing exponential growth of cancer cells and inhibiting the development of preneoplastic lesions) and can even cause the activation of apoptosis [14, 22] in these cells. *In vivo* studies showed that resveratrol inhibits the development of skin cancer, reducing tumor diameter and its incidence [6], induces cell apoptosis in human breast carcinoma and in human leukemia by conversion to piceatannol, an antileuketic stilbene [6, 22].

1.2.4. Anti-inflammatory activity

Effective even in micromolar concentrations, resveratrol may reduce the inflammatory endothelial cell damage caused by environmental toxicants [22]. During inflammatory response, resveratrol elicits inhibitory effects in all physiopathological phases, suggesting that resveratrol could be effective in pharmacotherapy [7]. In combination with its antioxidant properties, resveratrol also intervenes on enzyme systems involved in the synthesis of pro-inflammatory mediators, decreasing reactive oxygen species (ROS), which contributes to gastric ulcer healing and the reduction of colon injury [7].

1.2.5. Anti-ageing

One of the subjects which arouse greater interest nowadays is the delay of the ageing process. It has been described that resveratrol can interfere with specific enzymes involved in the mitochondrial metabolism, the sirtuins [27]. Sirtuins are a family of NAD^+-dependent deacetylases implicated in transcriptional silencing, ageing and metabolic regulation and are ubiquitous throughout all kingdoms of life, having a greater presence in evolutionarily superior creatures [21]. Resveratrol is a strong activator of sirtuin activity and applying these up-regulated enzymes to differentiated fat cells, lipolysis occurs, promoting longevity [7]. In another hand, sirtuins also play an important role in DNA repair processes and senescence, modulating the ageing process, slowing senescence and increasing lifespan, also controlled by resveratrol [14].

1.2.6. Other activities

Several benefits of resveratrol are already confirmed; however, many other effects are known, such cardio and neuroprotective activities. The areas of action are frequently interconnected, linking, for example, the antioxidant and anti-inflammatory properties with cardioprotective activities. This stilbene has a protective action against acute ischemic stroke, improves the motor performance and attenuates oxidative stress after ischemia reperfusion [25]. In addition, resveratrol protects the neurons [25], breaking down beta-amyloid aggregates – which are associated with Alzheimer's disease – and also up-regulates kinases which are active in the learning and memory centers of the brain [26]. Several neurologic benefits associated with Parkinson's disease, amyotrophic lateral sclerosis and brain edema were also described for this compound [6]. Furthermore, various studies revealed the protective effects of resveratrol against diet-induced obesity and insulin resistance [6].

1.2.7. Metabolism and bioavailability of resveratrol

The investigation of resveratrol metabolism in humans is quite recent. Some studies performed in rats, report that oral administration of resveratrol, formulated as solid lipid nanoparticles or nanostructured lipid carriers [28], for example, led to an important absorption [29] and was pharmacologically active both *in vitro* and *in vivo* [22].

After oral intake, resveratrol was absorbed quite rapidly whether as aglycone or on its glycosidic form; however, the levels of free resveratrol in plasma and serum were very low or no detectable [30]. Half of the resveratrol orally administrated to rats was absorbed from the digestive tract, especially in small intestine [29]. Resveratrol was distributed to various organs, accumulating mostly in the liver [29], where it was converted to its glucoronide and sulfate conjugates prior to elimination, restricting the *in vivo* bioavailability. No phase I reactions, the first phase of biotransformation that increases the polarity of the compound, were observed in all studied systems [31]. One of the major ways for resveratrol elimination was renal excretion, resulting in high levels of resveratrol conjugates in urine [30], revealing

preferential excretion of more polar forms [29]. Due to small amount of resveratrol found in feces, fecal route was thought to be a minor way of elimination [29].

1.2.8. Resveratrol toxicity

So far, there are no valid data on the toxicity of chronic intake of resveratrol by humans, and dosages in the range of hundreds of mg to several g per day have been proposed, based on animal pharmacological studies. To this date, the results obtained by various groups are not conclusive, since a clear relation between administered dose and response was not found [32].

Overall, it is known that low doses of resveratrol may have a protective effect in human health, while high doses of this compound may be harmful [33]. Nevertheless, data obtaining from animal trials are promising and indicate the need for further human clinical trials [34], in order to evaluate resveratrol effectiveness and toxicity towards a safe administration of this compound. With regard to get more data on the dosage and efficiency of resveratrol in human health, it is necessary to comprehend this stilbene metabolism and bioavailability.

1.3. Resveratrol production

Although few studies were performed in humans, the beneficial effects of resveratrol are already well known. Its properties are desirable for several industries and, therefore, it is important to study resveratrol production and develop new ways to achieve a sustainable production.

Besides pharmaceutical/nutraceutical market and cosmetic industry, resveratrol is starting to be explored as a multifunctional compound that could be used in a ubiquitous manner; for example, from functional wines to supplements. Resveratrol is commonly extracted/purified mainly from grapevine or chemically synthesized. However, in order to meet the market demand, it is necessary to accomplish alternative ways and/or improving existing pathways to produce this stilbene. Biotechnology can offer an opportunity to get this compound by using plant cell cultures (cell suspension, hairy root and callus cultures) or genetically-manipulated microorganisms (Table 2).

1.3.1. Resveratrol biosynthetic pathway

Resveratrol is a plant secondary metabolite derived from shikimate-phenylpropanoid and/or polyketide pathway [35]. The plant shikimate pathway has two end-products that are the entry to the biosynthesis of phenylpropanoids: phenylalanine and tyrosine [3]. Resveratrol is formed on the phenylalanine/polymalonate pathway, being the last step of this biosynthesis pathway, and can be synthesized either from phenylalanine or tyrosine. Both phenylalanine and tyrosine precursors produce *para*-coumaric acid (*p*-coumaric acid, also known as *para*-hydroxycinnamic acid) [6] (figure 2).

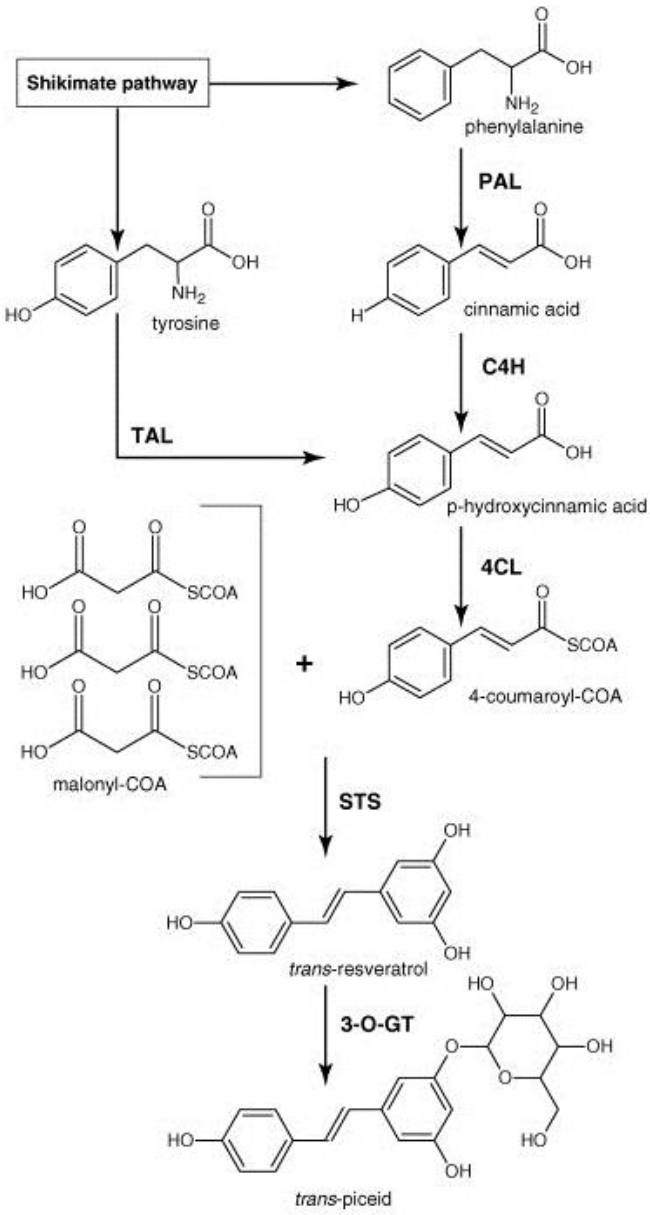


Figure 2. Resveratrol biosynthesis via phenylalanine/polymalonate pathway [9].

para-Coumaric acid is generated from phenylalanine through phenylalanine ammonia lyase (PAL) and cinnamate 4 hydroxylase (C4H), which acts in the cinnamic acid intermediate. Tyrosine ammonia lyase (TAL) exerts its activity directly in tyrosine [6]. Then, *para*-coumaric acid is activated by ligation to coenzyme A (CoA) by 4-coumaroyl:CoA ligase (4CL) and in the pathway-committing step, stilbene synthase (STS) condenses three units of malonyl-CoA (from fatty acids biosynthesis) with *para*-coumaroyl-CoA, forming a linear tetraketide molecule (not shown) before a cyclization reaction carried out by STS, generating resveratrol [16]. *trans*-Resveratrol can be modified to *trans*-piceid by 3-O-glucosyltransferase (3-O-GT) [9]. However, resveratrol has several alternatives to chemical synthesis, mainly production by plant cell cultures and using recombinant microorganisms (table 2).

Table 2. Resveratrol production systems.

System	Type of culture	Organism	Production levels	Advantages	Disadvantages
Plant cell cultures	Cell suspension cultures	*Gossypium hirsutum L.* (cotton)	0.0072 mg/g	Unnecessary genetic modification; Homogeneity of cell population; Good reproducibility; High cell growth rate; No contamination by microbial toxins or animal pathogens.	Dependence on plant species, elicitor and culture conditions; Require light; Costly purification.
		Vitis vinifera (grapevine)	5027 mg/L		
	Hairy root	*Arachis hypogaea* (peanut)	1.5 mg/g	Rapid growth; Production from a single line; Recover from culture media; Common and inexpensive medium; No contamination by microbial toxins or animal pathogens.	Large fermentator; UV radiation elicitor; Length of time; Costly purification.
	Callus cultures	*Arachis hypogaea* (peanut)	0.012 mg/g		
		Vitis vinifera (grapevine)	33 mg/g		
Microorganisms	---	*Escherichia coli*	2340 mg/L	Fast growth; Continuous fermentation; Low cost; Genetic tractability.	Industrial scale extraction and purification is difficult and expensive; cGMP required.
	---	*Saccharomyces cerevisiae*	391 mg/L	Food grade status; Fast growth; Economically profitable; No endotoxins or oncogenes; Contains malonyl-CoA.	

1.3.2. Resveratrol production by plant cell cultures

Plants are versatile organisms regarding to the production of various compounds. In this way, genetically-engineered plants could be an alternative to create functional food. Stilbene synthase genes were transferred to various crops, as tomato, rice, banana and lettuce [36]. Resveratrol engineering also led to increased resistance to the disease in transgenic plants: the introduction of two grapevine stilbene synthase genes in tobacco confers higher resistance to *Botrytis cinerea* infection [36]. However, genetically-engineering resveratrol to obtain crop defense against pathogenic microbes, improve nutritional value or to extend the shelf-life of the products may have a relatively huge cost. In addition, these products are distrusted by regulatory agencies and public; then, the commercial future of resveratrol engineering in plants remains to be seen [37]. Nevertheless, one commercial example was successfully demonstrated, by the production of red wine extracts and stilbene capsules [13].

Plant cells cultures have been an attractive source for the production of secondary metabolites and other substances of commercial interest. Because plant cells are biosynthetically totipotent, each cell in culture retains complete genetic information and hence is able to produce chemicals found in the parent plant [38]. Besides that, cultured plant cells proliferate *in vitro* indefinitely – unlike primary animal cells cultures that undergo only a limited number of cell divisions [39]. These systems have several other advantages, such as their independence of geographical and seasonal variations, performance of stereo and regiospecific biotransformations and an efficient downstream recovery [38].

The concentration of *trans*-resveratrol produced using plant cell cultures is, at least, equal to that reported as naturally occurring in the plant – which prevents intensive cutting and decimation of natural sources [9]. Furthermore, natural sources often contain derivatives, cofactors and other phytonutrients that provide added of synergistic benefits to the product [40]. There are two plant cell systems that are studied in particular: cell suspension cultures and hairy roots/callus [9]. Plant cell cultures are carried out under aseptic conditions and require a culture medium consisting of water, inorganic salts, sucrose, vitamins and plant hormones [39]. These two main plant cell systems are adaptable to scale-up for bioreactors [9].

1.3.2.1. Cell suspension cultures

Cell suspension cultures are an important tool for plant biology investigation. Due to homogeneity of an *in vitro* cell population, large availability of material, good reproducibility of conditions and high rate of cell growth, this technique is suitable to produce *trans*-resveratrol *in vitro* [9].

For this type of culture, the main advantage is the unnecessary genetic modification of the plant cell, because they are able to produce *trans*-resveratrol constitutively or in response to stress [9]. This last hypothesis can be exploited for *in vitro* resveratrol production by elicitation using several elicitors, such as chitosan [41], methyljasmonate (MeJa), ethylene [42], cyclodextrins (CDs) [43], as RAMED (Randomly Methylated-β-cyclodextrin) or DIMED (2,6-di-O-methyl-β-cyclodextrin) [9], sodium acetate, amino acids, sugar or UV-irradiation [9]. Apart from being elicitors, cyclodextrins also protects resveratrol in the medium by complex formation [9], leading to a significant production of resveratrol. Although MeJa affects cell growth and does not promote the increase of resveratrol, a combined treatment with MeJa and CD led to an increased accumulation of resveratrol in the medium [43], because they act synergistically, inducing the expression of STS and the general phenylpropanoid pathway, leading to a marked increase in the resveratrol amount [43]. Nevertheless, resveratrol production is affected by a wide range of factors, such as plant species, elicitor and culture conditions – resulting in fluctuating values in terms of response to elicitors [9].

Several plants have been used for resveratrol *in vitro* production. The most common for this purpose are *Vitis vinifera* (grapevine) and *Gossypium hirsutum L.* (cotton) [9]. In cotton,

where resveratrol is produced in low quantities (0.0072 mg/g) [9], synthesis may depend on cultivar, due to its appearance only in certain varieties [44]. On the other hand, some *Vitis vinifera* cell suspension cultures are particularly suitable for resveratrol production, due to the ability to produce piceid and several *trans*-resveratrol glucoside derivatives, which are easily hydrolysed by glucosidases [9]. However, this cultivation method for *Vitis vinifera* may require light, which becomes a limitation due to the isomerization of *trans*-resveratrol [9]. Grapevine cell suspension cultures are the main targets for the use of elicitors. *Vitis vinifera* cells under the influence of chitosan change their protein expression profile and stilbene distribution, leading to endogeneous accumulation of *trans*-resveratrol [41]. Without any elicitors, cell suspension cultures of *Vitis vinifera* produced 280 mg/L. However, when RAMED was added, the amount of resveratrol increased to 5027 mg/L [9]. Although this is the highest concentration of resveratrol obtained by any type of culture, it has been only carried out in a small laboratory scale [9].

Although they are a commonly used method for resveratrol cultivation, these systems may present some issues, as loss of secondary metabolite production after elicitation or genetically instability [40].

1.3.2.2. Hairy root and callus cultures

Callus cultures and especially hairy root cultures offer a novel and consistent system for the synthesis of bioactive secondary molecules [40].

Root development is performed using the bacterial plasmid Ri T-DNA or *rol* genes from *Agrobacterium rhizogenes* [9] and *Agrobacterium tumefaciens* [40]. Plant tissues are transformed by transferring the plasmids mentioned above, which causes a genetic modification in the plant material leading to the development of roots, able to grow in liquid media and to produce secondary metabolites [9]. However, the various strains used are not equally effective in genetic transformation (bacterial infection frequency) and subsequent root growth, as well as in stilbene production [9], and thus, *Agrobacterium rhizogenes* is largely selected to this technique [40].

Hairy root cultures are an alternative way to produce secondary metabolites owing to some inherent characteristics, such as rapid growth, genetic and biochemical stability and the ability to synthesize natural compounds as *in vitro* plants [45]. These tissue-based systems have the potential of producing various combinations of valued products from a single production line and also reflect accurately the performance and metabolic phenotype of the host plant, comparing to plant cell cultures [40]. In addition, resveratrol can be readily recovered from culture media and evidences suggest that hairy root cultures may be feasible in industrial processes [40].

In fact, resveratrol was successfully produced using hairy root cultures from *Arachis hypogaea* (peanut), resulting on a reliable, well-defined and easy method [45]. However, the yield of

resveratrol production by hairy roots is low: 1.5 mg/g [9]. The peanut plant, as well as grapevine, can also be used in callus cultures [45, 46]. Calli are coalescences of dedifferentiated cells that grow on a solid culture medium [9] and this is the starting point for the development of plant cell suspension cultures. *Vitis vinifera* was already used to produce resveratrol through callus cultures, where the largest amount achieved was 33 mg/g [9]. The basic requirements for this technique are a large fermentator, which may be a disadvantage, and a common and inexpensive medium at room temperature. Sometimes it is necessary to elicit the fermentation with UV radiation, which can be a disadvantage when producing resveratrol [46], because it may transform the isomer *trans* in *cis*, less important to human health, and the length of time needed for culturing cannot be economically profitable [9].

As seen before, plants are possible systems to produce resveratrol. Being a possible source of cheap recombinant compounds, an important advantage is that products from recombinant plants or plant systems are unlikely to be contaminated by microbial toxins, oncogenic sequences or animal pathogens [47]. Nevertheless, purifying transgenic compounds may be costly. An alternative to plant cell systems and an approach still under development is resveratrol production using microorganisms.

1.3.3. Resveratrol production by microorganisms

Several drug precursors and drugs are found naturally in organisms, but are difficult to synthesize chemically and to extract in large quantities. In this manner, metabolic engineering, which considers metabolic and cellular system as an entirety, is of utmost importance in the production of these compounds. Metabolic engineering allows performing changes at the whole cell level, enabling optimal design of a microorganism for the efficient production of compounds and biomolecules [48]. Metabolic pathway optimization has some key issues to be considered. One of the most important issues is the choice of the host strain and its capability to perform the desired metabolic conversions for product formation [48]. The host strain should be the most adapted to perform the desired metabolic conversions, considering that different organisms possess different metabolic capabilities [48]. The genetic and physiological backgrounds of the host are also causes of concern due to the importance of the appropriate intracellular environment and if it produces sufficient amount of precursors or redox compounds required for coordinate the genes [48]. The quality and quantity of the compound of interest also depends upon the expression system, as well as the bio-characteristics and purposes of the expressed products [49]. Furthermore, the convenience of an optimal expression system, availability and the overall cost should be taken in consideration when selecting a host system [49]. Once microorganisms are widely used to produce molecules, engineering bacteria or yeasts to produce resveratrol is a valuable mean for its production in large quantities [9]. Neither yeast nor bacteria should be tailored, because none of them possess the genes that encode for resveratrol pathway [9]. Thereby, there are two strategies for this purpose: introduce the entire pathway using as substrates L-

phenylalanine or L-tyrosine or introduce specific genes and use *p*-coumaric acid as a precursor [6, 9]. However, both in *Saccharomyces cerevisiae* and *Escherichia coli*, resveratrol accumulate naturally in the medium rather than in the cells, which is particularly advantageous considering commercial production system and its viability [18]. Despite these advantages, resveratrol extraction and purification in an industrial scale remain difficult and expensive due to the process compliance with cGMP (current Good Manufacturing Practices) requirements [9].

1.3.3.1. Resveratrol production by *Escherichia coli*

Escherichia coli is one of the most used host systems in biotechnology. This bacterium is frequently preferred for pharmaceutical production due to its fast growth, ability for continuous fermentation, relatively low cost [49] and well known genome, resulting in a vast array of genetic engineering tools available [48]. However, *E. coli* have been studied for resveratrol production (Table 3) because of its well characterized fermentation properties and genetic tractability [8].

Most bacterial genes are transcribed into messenger RNA (mRNA), which in turn is translated into protein. The structural gene encodes the gene product and its expression is controlled by sequences in the upstream region. Some proteins and RNA molecules are needed in the cell at about the same level under all growth conditions – the constitutive expression [50]. The expression is regulated and is a major process in all cells, helping to conserve energy and resources [50]. However, some enzymes are synthesized only when the substrate is present – inductive expression [50]. Constitutive gene expression allows to RNA polymerase to continue transcription [50] without any inhibition.

Resveratrol production from *E. coli* expression systems introducing the entire pathway using amino acids as substrates was already performed by genetic engineering of *E. coli* BL21 (DE3) strain, introducing PAL and TAL enzymes. However, TAL was only expressed when 20 mg/L of *p*-coumaric acid was added. This need for *p*-coumaric acid has a detrimental effect on enzymatic activity, leading to a low resveratrol production. Since introducing *de novo* the entire pathway has proved to be a very difficult procedure, an alternative route for resveratrol production in *E. coli* has been described, consisting in the transformation of *E. coli* with 4CL and STS genes with the use of 4-coumaric acid as precursor for resveratrol production [9].

E. coli BL21 (DE3), with STS and 4CL genes, was able to produce 3.6 mg/L of resveratrol [9]. *E. coli* BL21 engineered with 4CL gene from tobacco and STS gene from grapevine produced 16 mg/L [9] and in another study it yielded up to 20 mg/L of resveratrol [18]. The BW22784 strain transformed with 4CL gene from *Arabidopsis thaliana* and STS gene from *Arachis hypogaea* was able to convert 4-coumaric acid into resveratrol at the maximum of 104.5 mg/L [51]. The STS gene used in the previous strains was constitutively expressed from the *lac* promoter [51]. The genes used in the previous strain were transformed in *E. coli* JM109,

which led to a production amount higher than 100 mg/L [9]. When the JM109 strain was transformed with 4CL gene from *Lithospermum erythrorhizon* and STS gene from *Arachis hypogaea*, coumaroyl-CoA was used as substrate and the production reached 171 mg/L [8]. Alternative gene expression was already tested to *E. coli* DHIOB. Having tyrosine as precursor, three genes were added: PAL from *Rhodotorula rubra,* 4CL from *Streptomyces coelicolor* and STS from *Arachis hipagaea*. This strain was able to produce 40 mg/L of resveratrol [8]. Another study [16] explored multiple constructs for resveratrol production by varying the 'construct environment'. Using 4CL gene from *Arabidopsis thaliana* and STS gene from *Arachis hypogaea* in *E.coli* BW27784 and an operon produced 404 mg/L. The same strain, with the same 4CL gene and a STS gene from *Vitis vinifera* produced 1380 mg/L. The values depend not only on production strategies but also from resveratrol supplements, which may be added to the medium and improve resveratrol production, as cerulenin, which was added to the same strain and genes from the last example. The result was a production of 2340 mg/L [16].

1.3.3.2. Resveratrol production by *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a unicellular organism that satisfies the biosafety regulations and the economic efficiency, reasons that explain why *Saccharomyces* has been engineered over time [49]. Being eukaryotic, yeasts share biochemical, molecular and genetic features with higher eukaryotes, which make it desirable for large-scale industry fermentation and with commercial potential, once it can perform the correct protein folding with all the post-transcriptional modifications [49]. Like *E. coli*, this yeast also grows rapidly (sometimes into high densities) and in simple media, which lowers the production cost [49]. In addition, this is a safe system, not containing endotoxins or oncogenes, and it is relatively easy to manipulate [49].

Beyond this, *S. cerevisiae* is a favored organism to produce resveratrol – because one of the precursors, malonyl-CoA, is already found in yeast [8]. The other resveratrol substrate is *p*-coumaroyl-CoA, resulting from *p*-coumaric acid, that has been shown to be accumulated by yeast [52]. Engineering the entire pathway for resveratrol production in yeast was not successful. Although the strain CEN.PK113-5D was modified in this way, resveratrol synthesis did not occurred [9]. Nevertheless, several studies about resveratrol production were performed using *S. cerevisiae*, and the results are condensed in table 3. A FY23 strain was modified with 4CL216 gene from a hybrid poplar and STS gene from grapevine produced about $1.5x10^{-3}$ mg/L of resveratrol [52]. The same strain was transformed with 4CL (*Populus trichocarpa* x *Populus deltoids*) and STS (*Vitis vinifera*) genes and was used *p*-coumaric acid. This work obtained a final amount of 1.45 mg/L of resveratrol [6]. The strain WAT11 was transformed with TAL (*Rhodobacter sphaeroides*), 4CL (*Arabidopsis thaliana*) and STS (*Vitis vinifera*) genes and produced 0.65 mg/L of resveratrol [6]. In another study, the previous strain was able to produce 1 mg/L of resveratrol [9]. Another strain – W303-1A – was transformed with 4CL1 gene from *Arabidopsis thaliana* and STS gene from *Arachis hypagaea*, a combination that has given positive results in *E. coli* [15]. In this case, resveratrol

production was about 3.1 mg/L [15]. In another study, 4CL2 gene from tobacco and STS gene from grapevine were transformed into CEN.PK113-3b strain which yielded 5.8 mg/L of resveratrol [8]. Another study [6] refers that an industrial yeast *S. cerevisiae* CEN.PK2-1 transformed with 4CL1 gene from *Arabidopsis thaliana* and STS gene from *Vitis vinifera* produced 391 mg/L of resveratrol, the highest amount produced for this yeast.

In an alternative approach, gene expression was used in *S. cerevisiae* RESV11, where phenylalanine was the substrate and five genes were transformed into this strain: PAL and CPR from poplar hybrid (*Populus trichocarpato x P. deltoids*), C4H and 4CL from *Glycine max* and STS from *Vitis vinifera*. This experiment resulted in a resveratrol production of $3.1x10^{-1}$ mg/L [8]. The results presented depend not only on the origin of the transferred genes, precursors and growth factors added [8], but also on the spatial localization of the enzymes and the provenance of the species and strains [6]. Although expression system selection is a crucial step when designing a production process, one should also consider the effects of external conditions such as growth medium and conditions, since they can have a great impact on overall production.

Table 3. Examples of recombinant expression systems used for resveratrol production.

Microorganism	Strain	Introduced genes	Production levels	References
Escherichia coli	BL21 (DE3)	4CL (*S. coelicor*), STS (*Vitis* sp)	3.6 mg/L	[9]
	BL21	4CL (*N. tabacum*), STS (*Vitis vinifera*)	20 mg/L	[18]
	BW22784	4CL (*A. thaliana*), STS (*Vitis vinifera*)	2340 mg/L	[16]
	DHIOB	PAL (*R. rubra*), 4CL (*S. coelicor*), STS (*A. Hypogaea*)	40 mg/L	[8]
	JM109	4CL (*L. erythrorhizon*), STS (*A. hypogaea*)	171 mg/L	[8]
Saccharomyces cerevisiae	CEN.PK113-3b	4CL2 (*N. tabacum*), STS (*Vitis vinifera*)	5.8 mg/L	[18]
	CEN.PK2-1	4CL1 (*Arabidopsis thaliana*) STS (*Vitis vinifera*)	391 mg/L	[6]
	FY23	4CL216 (*Populus trichocarpa* x *Populus* deltoids), STS (*Vitis vinifera*)	$1.5x10^{-3}$ mg/L	[52]
		4CL (*Populus trichocarpa* x *Populus deltoids*), STS (*Vitis vinifera*)	1.45 mg/L	[6]
	RESV11	PAL, CPR (*Populus trichocarpato x P. deltoids*), C4H, 4CL (*Glycine max*) STS (*Vitis vinifera*)	$3.1x10^{-1}$ mg/L	[8]
	WAT11	TAL (*Rhodobacter sphaeroides*), 4CL (*Arabidopsis thaliana*), STS (*Vitis vinifera*)	1 mg/L	[9]
	W303-1A	4CL1 (*Arabidopsis thaliana*), STS (*Arachis hypagaea*)	3.1 mg/L	[15]

1.3.4. Cultivation of microorganisms

Different organisms have different nutritional necessities. Macronutrients, such as carbon, hydrogen, oxygen, nitrogen, phosphorus and sulfur, are essential and required in large amounts as they constitute proteins, nucleic acids, lipids and polysaccharides [50]. Micronutrients, such as iron, manganese and other trace metals, as well as growth factors and vitamins, are required in very small amounts, which function as coenzymes [50].

With respect to its composition, culture media can be classified in defined, semi-defined and complex. In a defined medium, all components composition and concentration is known whereas in complex media, the composition of some components is variable and unknown. This last type of media usually contains digests of microbial, plant or animal products and other highly nutritious yet impure substances, commercially available in dehydrated form but the exact nutritional composition is unknown [50]. The intracellular environment is a reflex of the extracellular media, and, in this way, the culture medium needs to be optimized, because it is highly associated with cell growth and, consequently, with the cost-competitiveness in the bioprocess [48, 49].

Once a culture medium is made and sterilized, it can be inoculated with a pure culture and then incubated in favorable growth conditions, which without outside interferences, is a batch growth [50]. Batch growth is constituted by four phases: lag, exponential, stationary and cell death phases. Lag phase, the first, occurs after the transference of inoculum from another culture to a new one and for this reason, cell require time to biosynthesize essential constituents [50]. The next phase is the exponential phase, where cells are healthy and exponential growth occurs, followed by the stationary phase. This phase happens before an essential nutrient is used up and/or a waste product accumulates in the medium, which inhibits cell growth. The last phase is bacterial death, sometimes accompanied with cell lysis [50].

Nonetheless, in order to perform fermentation studies, shake-flask cultures are widely used [53]. Utilized for more than two centuries, shake flasks cultures are employed to select suitable strains for production scale. Shake-flask cultures are small-scale cultures performed using liquid medium and incubated under conditions that favor growth. In this manner, it has become increasingly important to study the cultivation parameters to optimize growth conditions [53]. The optimum conditions for cell physiology and metabolism are regulated by various chemical and physical factors. Temperature is one of the most important physical factors to microorganisms. Temperature affects microorganisms in two opposing ways and three temperatures, called cardinal temperatures, are characteristic for any microorganism and encompass minimum, maximum and optimum points [50]. At minimum temperature, transport processes are very slow and growth cannot occur; besides, membrane gelling occurs. As the temperature rises, optimum point is achieved and enzymatic reactions occur at maximal possible rate. Optimal point is near to maximum temperature, were protein

denatures, the cytoplasmic membrane collapse and thermal lysis occurs. In the extreme points, growth is unviable [50]. Resveratrol is affected by temperature, as showed in a study [54], which underwent resveratrol by heat treatment, resulting in a decreased content of resveratrol under temperature stress (35 ºC). pH is another important element in microorganisms growth. The most natural environments have a pH between 4 and 9, and organisms with optimal pH in this range are most commonly encountered [50]. Resveratrol is also affected by different pH, as demonstrated in a study performed by [55], where resveratrol is stable until an approximately pH of 9, and then starts to deprotonate.

Oxygen can be a requirement for microorganisms, but is not so essential as an adequate temperature and pH [50]. Many organisms can live in the total absence of oxygen; however, *E. coli* and *S. cerevisiae* are aerobes. Resveratrol can be affect by oxygen dissolution over a long period of time, and a study about resveratrol stability in wine storage revealed that over time, the amount of resveratrol decreased significantly [56]. These growth conditions can be controlled when the fermentation is performed in a bioreactor, enabling a higher production of the compound of interest [57].

The most widely used bioreactor is stirred tank bioreactor. This bioreactor has an easy scale-up, good oxygen transfer ability as well as good fluid mixing. It also has alternative impellers and easy compliance with cGMP [57]. However, it has some disadvantages, as high power consumption and high shear [57].

An effective bioreactor should provide a high volumetric productivity [57]. The yield depends not only on the bioreactor but also the bioreactor operation. The batch culture is one of the most known. In this type of operation, an organism grows in an enclosed vessel but the imposed conditions remain constant only in the early stages of exponential growth, because there are no toxic metabolites in media or depletion of nutrients [50]. Nonetheless, in the later stages, the number of cells increases and, consequently, the physical and chemical composition of the medium changes and thereby affects both growth rate and growth yield [50]. In this manner, although batch cultivations are known by constantly changing its environmental conditions, this method is capable of produce metabolites associated with any kinetic pattern [58].

1.4. Statistical design of experiments

Since many factors can play an important role in a fermentation process, several approaches have been described in order to optimize this process towards a more successful production. Sometimes, these experiments can be a discussed in a one-factor-at-a-time approach. But this methodology is time consuming, does not consider any possible interaction between factors and does not consider the failure of one factor to produce the same effect on the response at different levels of another factor [59]. As a response for these cases, experimental design has emerged.

The one-factor-at-a-time approach is a strategy of experimentation that is used extensively in practice. After selecting a starting point (or a baseline set of levels, for each factor), each factor is then successively varyied over its range with the other factors held constant at the baseline level [59]. After carry out all the tests, several graphics are constructed, showing how the response variable is affected by varying each factor with the others constant, which are used to select the optimal combination [59]. Because this approach does not take into account the possibility of interaction between factors, which can lead to incorrect conclusions, a more accurate approach is to conduct a factorial experiment, where factors vary together instead of one at a time [59].

Design of Experiments (DoE) is an approach to study several factors from a particular process. Experiment could be defined as series of tests where changes are performed to the input variables and then output response is observed and reasons of changes are identified [59]. This methodology is an important tool for improving the performance of a manufacturing process, or in the development of new processes. Experimental design could lead to improved process yields, reduced overall costs, development time and variability [59]. However, factorial designs have further advantages: are more efficient than one-factor-at-a-time experiments, the presence of interactions avoid misleading conclusions and this type of approach allow the effects of a factor to be estimated at several levels of the other factors, yielding conclusions that are valid over a range of experimental conditions [59].

The design of experiments could follow seven steps. The first step is the recognition and statement of the problem, the next step is choosing the factors, levels and ranges, also selecting the response variable. Once these steps are done, the next is choosing the experimental design. Then, is necessary to perform the runs and hereafter analyze statistically the data obtained, resulting in valid and objective conclusions and remarks [59]. The choice of a particular design involves the consideration of the number of replicates and a suitable run order. At the end, once the data was analyzed, follow-up runs and confirmation testing should be performed to validate the conclusions from the experiment [59].

There are three basic principles on design of experiments. Replication is one of them. This term is related to repetition of the basic experiment, allowing the achievement of an estimate of the experimental error [60]. It is also used to determine whether observed differences in data are statistically different and to estimate the effect of a factor in the experiment. Replication reflects variability between runs and within runs [59]. Another basic principle is randomization. It is related to the order in which the individual runs or trials of the experiment are performed, randomly determined. This randomization is necessary as a requirement of statistical methods and the effects of extraneous factors may be noted [59]. Blocking is the last basic principle. This principle is a design technique to improve precision and reduce or eliminate the variability from nuisance factors (which may influence the experimental response but is not the focus of interest). In order to optimize processes with

combination of several independent variables and correspondent interactions, an effective tool is Response Surface Methodology (RSM) [61].

1.4.1. Response Surface Methodology (RSM)

Response Surface Methodology quantifies the relationship between controllable input parameters and obtained response surface [60]. The main objective is to optimize the response surface that is influenced by various process parameters.

The design procedure to perform RSM starts with the design of a series of experiments for adequate and reliable measurement of the response of interest. Then, it is necessary to develop a mathematical model and determine the optimal set of experimental parameters. Finally, the direct and interactive effects of process are represented in two and/or three dimensional plots [60].

The response surface can be resumed in an equation if all variables are measurable [60]:

$$y = f(x_1, x_2, x_3, \dots, x_n) \quad (1)$$

Where y is the response of the system and x_i the variables of action called factors. The main goal of this methodology is to optimize y.

In order to optimize y, three types of experimental design techniques are usually used for process analysis and modeling: full factorial, partial factorial and central composite rotatable [60]. The first type requires at least three levels per variable to estimate the coefficients of the quadratic terms in the response model. The second type is particularly useful if certain variables are already known and requires fewer tests than full factorial [60]. The third type is able to give large amounts of information but with a relative reduced number of experiences.

1.4.2. Central Composite Rotatable Design (CCRD)

This methodology was developed by Box and Wilson and afterwards improved by Box and Hunter. This method gives almost as much information as a three-level factorial, but is sufficient to describe the majority of process responses and requires fewer tests than full factorial design [60].

As seen before, a factor could have levels and ranges. When there are k factors, each at two levels, factorial design would require 2^k runs [59]. This kind of design includes 2^k factorial with its origin at the center, $2k$ points fixed axially at a distance (β) from the center to generate the quadratic terms and replicate tests at the center [60]. The axial points are important because they allow rotatability, ensuring that the variance of the model prediction is constant at all equidistant from the center points [60]. A representative scheme of central

composite rotatable design is presented in figure 3. The number of tests recommended for four variables at the center is six [62].

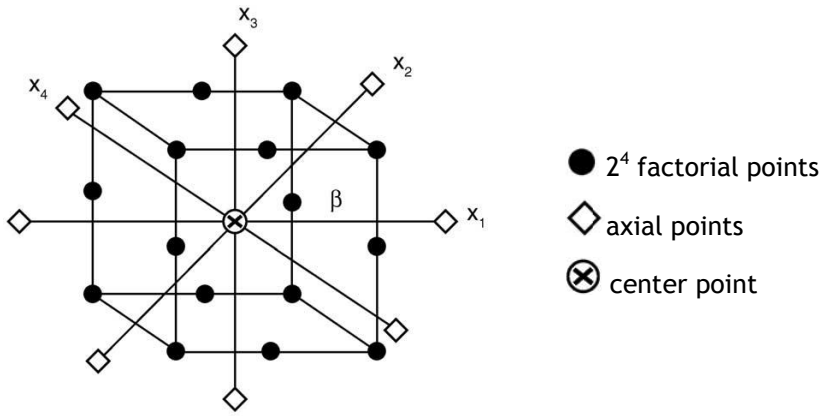


Figure 3. A CCRD scheme for four factors x_1, x_2, x_3 and x_4 (Adapted from [63]).

When the desired ranges of values from the variables are chosen, points are coded as functions of the range of interest. So, factorial points are coded as ±1, central points as 0 and axial points are $\pm\beta$ [62] (table 4).

Table 4. Relation between coded and actual value of variables [62].

Coded points	
Code	Actual value of variable
$-\beta$	x_{min}
-1	$\left[\frac{x_{max} + x_{min}}{2}\right] - \left[\frac{x_{max} - x_{min}}{2\alpha}\right]$
0	$(x_{max} + x_{min})/2$
$+1$	$\left[\frac{x_{max} + x_{min}}{2}\right] + \left[\frac{x_{max} - x_{min}}{2\alpha}\right]$
$+\beta$	x_{max}

When data is obtained, a regression analysis is carried out to determine the coefficients of the response model, significance and standard errors. In this manner, response model incorporates constant (a_0), error (ε), linear terms of each variable ($x_1, x_2, \ldots, x_n$), squared terms of each variable ($x_1^2, x_2^2, \ldots, x_n^2$) and first order interaction terms for each paired combination ($x_1x_2, x_1x_3, \ldots, x_{n-i}x_n$). So, the general final equation of response model is [60]:

$$y = (a_0 + \varepsilon) + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii}\, x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} b_{ij} x_i x_j \qquad (2)$$

Also, ANOVA (analysis of variance) is performed to obtain a detailed statistical significance of the linear, square and interaction components of the model above. The value of the model allows the prediction of the performance by interpolation, identifies statistically different significant factors and facilitates the construction of response surface graphics [63].

After optimal production in bioreactor or shake-flasks, it is necessary to recover and quantify the resveratrol produced through a chromatographic method.

1.5. Resveratrol recovery and quantification

Although some samples can be analyzed by direct injection, generally a sample preparation is made prior to injection, as a pre-concentrate and cleaning stage [64]. For the recovery of resveratrol from culture media, several extraction processes (Table 5) have been described such as liquid-liquid extraction (LLE), solid phase extraction (SPE) and solid phase microextraction (SPME) [17]. Nevertheless, LLE is the most widely used method for resveratrol recovery, because it is simple, robust, can use a wide range of organic solvents and extractants, efficient and widely accepted in many standard methods [65]. After the extraction, the samples are concentrated by evaporation to dryness and re-dissolved in chromatographic solvent, followed by chromatographic injection [66].

Table 5. Extraction techniques for resveratrol extraction from complex matrices.

Type of Extraction	Description	Advantages	Disadvantages	References
Liquid-Liquid (LLE)	Separates components dissolved in a solution by contact with a second liquid phase.	High selectivity; Versatile; Cost-effective; Low energy consumption.	Use high purified solvents; Time-consuming.	[65, 67, 68]
Solid Phase (SPE)	Uses a solid phase and a sample matrix to isolate an analyte from a solution.	High selectivity; Easy automation; Low cost; High recovery and purity; Reduction of organic solvent.	Formation of carbon dioxide (CO_2) could disturb the efficiency; Efficient than LLE.	[64, 69, 70]
Solid Phase Microextraction (SPME)	With the same base than SPE. The analyte is desorbed at high temperature.	Decrease the use of organic solvents; Simple; Low sample volume; Higher detection limits than SPE.	Narrow application; Efficient than LLE.	[67]

For resveratrol quantification in various types of samples, several methods have been developed (Table 6), and the most are of a chromatographic nature. The majority of the

techniques used are liquid chromatography coupled to various types of detectors, such as UV, fluorescence or electrochemical detectors and even to a mass spectrometer [17]. Gas chromatography is also widely used, generally coupled to a mass spectrometer [64]. Non-chromatographic methods are used, but in a smaller amount of analysis, being capillary electrophoresis the most known method [17]. The remaining techniques (differential pulse voltammetry [71], laser desorption coupled resonant ionization spectrometry [72] and UV spectrophotometric detection [73]) are slightly used and they are still in an initial stage. The methods used are described in Table 6.

Table 6. Analytical procedures for resveratrol quantification.

Chromatographic Techniques			Detector	References
Chromatographic Techniques	Liquid	High-Performance Thin-Layer Liquid Chromatography (HPTLC)	UV, electrochemical, fluorescence (FLD), chemiluminescent, Mass Spectrometry (LC-MS)	[17, 64, 72]
		(Ultra) High-Performance Liquid Chromatography ((U)HPLC)		
	Gas	Gas Chromatography (GC)	Mass Spectrometry (GC-MS)	
Non-Chromatographic Techniques	Capillary Electrophoresis (CE); Differential Pulse Voltammetry; Laser desorption coupled resonant ionization spectrometry; UV spectrophotometric detection.			[17, 71-73]

Understanding the bioprocess is important to optimize and achieve maximal exploitation of the microorganism. Several techniques can be used to analyze the critical variables of the process and they should be constantly monitored. In this way, flow cytometry can be used to assess the physiological states of the cell, one of the most important issues in terms of any production process [74], since viability is compromised, cells are no longer able to grow and provide energy for product formation, resulting in a decrease in product yields.

1.6. Cellular viability

Rapid detection of microorganisms and the assessment of their viability can be an important tool to control bioprocesses [75]. This data is also relevant to design an effective production process, once a presence of an elevated number of dormant or dead cells may decrease product synthesis [74]. Flow cytometry allows the characterization of intracellular content in individual cells and cellular functions, where are included the assessment of membrane permeability and membrane potential, cell size, enzyme activity and total DNA and RNA contents [74]. The analysis of the physiological states of a cell population is of utmost importance to assess the impact of the culture conditions and length of fermentation, growth development and resveratrol production of a cell population. The rapid analysis of single cells in a mixture, versatility and automated sample handling for an increased sample throughput

make flow cytometry (FC) a desirable technique that has impacted biotechnology [75]. Flow cytometry is a quantitative technique that analyzes cell populations at single cell level. A single stream of particles formed by the hydrodynamic focusing passes through laser beam, measuring a large number of particles while simultaneously measure several parameters on each cell, separating them into populations, detecting minority or rare cells [75]. This technique can be performed using only scattering signals. In this way, cell size and complexity are evaluated through forward (FSC) and side (SSC) scatter light measurements [74]. While FSC assesses the size of the cell, SSC evaluates the complexity. However, in order to analyze the physiological state and viability, several fluorescent stains were developed and, nowadays, several multi-staining procedures can be performed. To assess membrane permeability, the most commonly used fluorescent stain is propidium iodide (PI). This fluorescent dye assesses bacterial membrane integrity; since, when the membrane is injured, PI is able to enter the cell and bind to nucleic acids, increasing the red fluorescence of cells [76, 77]. In order to evaluate bacterial membrane potential, bis-(1,3-dibutylbarbituric acid)trimethine oxonol (BOX) is commonly used. BOX has high voltage sensitivity and is an anionic and lipophilic molecule, capable of entering the cells but is readily excluded when cells are polarized. However, if cells enter a depolarization state, BOX can enter and accumulate inside the cells. Membrane depolarization is transitory and reversible in bacterial cells as, under appropriate conditions, depolarized cells can regrow and return to their polarized state [77]. This technique informs about heterogeneity, a characteristic present in all living organisms populations [74]. Flow cytometry arises as a tool that provides an insight into bioprocesses by evaluating several parameters.

1.7. Plasmid stability

Plasmid stability is also a key issue in recombinant microbial fermentations, because it may be present some plasmid-free segregants, even when pressure is exerted [78], which contributes to a diminished final plasmid DNA yields and consequently leading to lower resveratrol production [74].

Generally, plasmids are used as vehicles to carry foreign genes into a host cell, being a widely used system to produce many compounds of interest, carrying also some metabolic burden [74]. During fermentation process, and even under selective pressure, some plasmid-free segregants may arise, as a result of the leakage of the selective gene product into the media [74]. Segregational plasmid instability could be described as the loss of plasmids due to irregular distribution of plasmids during cell division [74]. Plasmid stability is determined by plasmid load, replication patterns, plasmid copy number, level of expression, substrate type and media composition and culture conditions [74]. Plasmids stability can be caused by the accumulation of plasmid multimers, leading to a decrease in the number of cellular units [74]. Although instability could impact downstream processes [74], this also impacts the

upstream process, since plasmid instability demonstrates a considerable decrease in plasmid productivity [74], which affects compound production.

However, there is another type of plasmid instability: the plasmid structural instability, which may arise from delections, insertions, duplications, inversions and translocations [74]. There are other factors that affect plasmid structural stability, as plasmid size, but also environmental stresses, as antibiotic concentration, medium composition, temperature shifts and oxygen fluctuations, elevating the rate of spontaneous point mutations [74]. Structural instability in plasmid could also be a result of the increased metabolic burden [74].

The stability of plasmid can be evaluated quantitatively, demonstrating that the plasmid copy number (PCN) of the recombinant plasmid is stable within the host cells [79]. PCN can be defined as the ratio between numbers of plasmid and chromosome amplicons at the moment of sampling [80] and may be quantified in an absolute or relative way [79]. PCN can be assessed by indirect and direct methods [74]. Indirect methods determined PCN by measuring the activity of the reported protein coded by the plasmid and normalized to the number of bacteria [80]. Direct quantification methods include cesium chloride (CsCl) centrifugation, southern blot hybridization, HPLC, capillary electrophoresis and agarose gel electrophoresis [80]. This direct quantification is based in the quantification of chromosomal and plasmid DNA followed by the calculation of the ratio between them [74].

Determine PCN during fermentation is a method to monitor plasmid segregational stability [74]. The results obtained by relative and absolute quantification are nearly equal; consequently, both methods can be applied to assess PCN in fermentations [74]. Because direct quantification methods require a DNA extraction procedure, which may lead to an inaccurate PCN determination, another method was develop to asses PCN by using whole bacterial cells [74]. This method provides a feasible approach for the time-course monitoring of PCN, once it only requires sampling and, dilution to maintain cell density at a constant value [74].

Real-time quantitative PCR (qPCR) offers a reliable, sensitive, precise and fast method for plasmids quantification in a sample [79]. Real-time qPCR is widely used to assess plasmid stability in bioprocesses. Sometimes, this technique is coupled to flow cytometry in order to determine cell physiological states and both are used to monitor fermentations. An example of the combined use of these techniques is performed by [81], were several plasmid DNA induction strategies were described and real-time qPCR and flow cytometry were used to determine pDNA segregational stability and cell physiological states during plasmid induction. This study concludes that DNA replication and cell filamentation occurs, two conclusions only possible through the use of real-time qPCR and flow cytometry [81].

The tools described here could be used to monitor and optimize the bioprocess and enable a better exploitation of fermentations and microorganisms. These tools should be used to

optimize the bioprocess and enable a better exploitation of fermentations and microorganisms.

1.8. Objectives

Resveratrol has a wide range of applications in several areas. The interest in resveratrol is increasing and it is necessary to accomplish the growing demand of industries for great amounts of this phytoalexin with economic value. The general goal of this study was the production of resveratrol in recombinant microorganisms using bioreactors, while monitoring cell physiology and plasmid segregational stability towards the improvement of bioprocess performance. The specific aims were:

- To validate a HPLC-DAD method for resveratrol quantification in culture media samples.
- To assess the influence of culture conditions and medium composition on resveratrol production in shake flasks using two recombinant microorganisms: *Escherichia coli* and *Saccharomyces cerevisiae*.
- To optimize resveratrol production through Design of Experiments (DoE) in bioreactors, evaluating cell physiology and plasmid stability.

Chapter 2

2. Materials & Methods

2.1. Transformation

2.1.1. Strains and plasmid

pAC-4CL1 plasmid (Addgene, USA) is a plasmid which encodes for 4-coumaroyl CoA ligase 1 from *Arabidopsis thaliana* and confers bacterial resistance to chloramphenicol. pUC-STS plasmid (Addgene, USA) is a high copy plasmid that encodes for stilbene synthase from *Arachis hypogaea* and confers bacterial resistance to ampicillin. *Escherichia coli* BW27784 (*E. coli* Genetic Stock Center, USA) was the selected host strain for the transformation with these two plasmids (pAC-4CL1 and pUC-STS). *Saccharomyces cerevisiae* FY23 was already transformed with vst1 and 4CL216 genes (FY23vst14CL216) and was kindly donated by Professor Maret du Toit from Stellenbosch University, South Africa.

In order to obtain pure plasmid for subsequent transformation, lysis and plasmid recovery was performed using a NZY Miniprep Kit (NZY Tech, Portugal) according to the manufacturer's instructions. An electrophoresis was performed as explained in section 3.1.2. Pure plasmids were stored at – 80 ºC.

2.1.2. *Escherichia coli* transformation

Escherichia coli was genetically manipulated using transformation by heat shock protocol.

Chemically, competent cells were generated using a method based in magnesium and calcium chloride addition. Exponentially-growing *E. coli* cells were obtained from a liquid culture in LB (Appendix 1) medium until the optical density at 600 nm (OD_{600}) reached 0.9. Then, a dilution was performed to a final OD_{600} of 0.05 in fresh LB medium and cultivated at 37 ºC, 250 rpm, to a final OD_{600} of 0.8. After, the culture was centrifuged at 5000 rpm for 10 minutes at 4 ºC. Cell pellet was resuspended in 12.5 mL of $MgCl_2$ 100 mM. The mixture was centrifuged at 4000 rpm for 10 minutes at 4 ºC and the resulting pellet was resuspended by inversion in 25 mL of $CaCl_2.H_2O$ (Panreac, Spain) 100 mM. This cellular suspension was incubated on ice for approximately 25 minutes and then centrifuged at 4000 rpm for 10 minutes at 4 ºC. Supernatant was carefully removed and the pellet was resuspended in 1 mL of $CaCl_2.H_2O$ 85 mM with glycerol 15 % (v/v) (HiMedia, India). This competent cell suspension was aliquoted and froze immediately in liquid nitrogen and stored at – 80 ºC. The transformation protocol is described below. After gentle thawing of the competent cells at room temperature, 1 µL of each plasmid (pAC-4CL1 and pUC-STS) was added to the cell suspension and incubated on ice for 30 minutes. Then, this mixture was heated at 42 ºC for 30 seconds and quickly transferred to ice. Afterwards, 200 µL of SOC (Appendix 1) medium was

added to the previous suspension and incubated at 37 ºC, 250 rpm for 2 hours. For selection of transformants, this suspension was spread in LB plates containing 50 µg/mL chloramphenicol (Fluka, USA) and 100 µg/mL ampicillin (Sigma-Aldrich, USA). As a control of the competent cell suspension, LB plates with antibiotics (ampicillin and chloramphenicol) were inoculated with an aliquot of the thawed competent cell suspension.

2.1.3. Cell lysis and electrophoresis

In order to verify if the *E. coli* strain was successfully transformed with both plasmids, plasmid DNA was extracted from liquid cultures in LB medium supplemented with ampicillin and chloramphenicol grown overnight at 37 ºC and 250 rpm. Cell lysis and plasmid recovery was performed using a NZY Miniprep Kit according to the manufacturer's instructions. A digestion was performed using Hind III Kit (NZY Tech, Portugal), according to the kit instructions. Agarose gel electrophoresis was performed using a 1 % agarose gel with Green Safe (0.2 µL/mL). Electrophoresis was carried out in Tris-Acetate-Ethylene Diamine (TAE) at 110 V for 45 minutes. The agarose gel was revealed under UV light and using 'UVITEC' software, UK.

2.1.4. Cell banking

Cell banks were made from *E. coli* and *S. cerevisiae* transformed cells. After overnight growth on LB plates, a single *E. coli* colony was placed into 25 mL LB medium, supplemented with chloramphenicol and ampicillin, and placed to grow at 37 ºC, 250 rpm until and OD_{600} of 0.6. In the case of *S. cerevisiae*, the media used was SC (Appendix 1) and yeast grew up until OD_{600} of 0.6 at 30 ºC, 250 rpm. Cells were cryopreserved in 30 % glycerol and stored at – 80 ºC.

2.2. Resveratrol quantification

2.2.1. Standards

Stock solutions (1 mg/mL) of carbamazepine (internal standard - IS) and *trans*-resveratrol (all purchased from Sigma-Aldrich, USA) were prepared by dissolving each pure substance in methanol HPLC-grade (VWR, USA). Carbamazepine was selected as internal standard because we could use the same extraction, chromatographic conditions and detection wavelength as used to resveratrol [17]. Working solutions of carbamazepine and resveratrol (1, 10 and 100 µg/mL) were made by proper dilutions in mobile phase. The solutions were stored at 4 ºC avoiding exposure to direct light.

2.2.2. Extraction procedure

Either in bacteria or in yeast, resveratrol is mostly accumulated in extracellular environment, which facilitates the recovery process [18]. After an optimization of the extraction procedure, the final conditions were as follows. 1 mL of culture media was centrifuged for at 13000 rpm for 5 minutes at 4 ºC. Supernatant was withdrawn to an extraction tube and the sample was acidified through the addition of 50 µL of HCl (VWR, USA) 1M, and the mixture was spiked with 50 µL of IS solution (100 µg/mL). This acidified sample was incubated in a

roller mixer at room temperature and low speed for 15 minutes, followed by the addition of 1 mL of ethyl acetate (Fisher Scientific, USA). The sample was incubated once again in a roller mixer at room temperature and low speed for 5 minutes. The organic phase was removed and afterward dried at 30 ºC under nitrogen stream. The dry extract was dissolved in 100 µL of mobile phase and subsequently filtered with a 0.22 µm pore size filter (Millipore, USA). About 50 µL were injected into Ultra-High Performance Liquid Chromatography system (UPLC), an Agilent 1200 series from Agilent Technologies, USA. The whole procedure was carried out in subdued light in order to avoid *trans*-resveratrol isomerization to *cis*-resveratrol during sample handling.

2.2.3. Chromatographic and detection conditions

The mobile phase was mixture of ultrapure water (Milli-Q system, Millipore, USA), acetonitrile (VWR, USA) and acetic acid (Fluka, USA), in a proportion of 66/33.9/0.1 (v/v/v) and pH 3.4 [17]. The solution was filtered through a 0.22 µm pore size membrane, degassed ultrasonically and pumped in isocratic mode through chromatographic system at 1 mL/min, with a column temperature of 25 ºC. Chromatographic separation was attained using a 5 µm Zorbax 300SB-C18 reverse-phase analytical column (4.6 mm ID x 150 mm) from Agilent Technologies, USA. The eluate was monitored at three different wavelengths using a Diode Array Detector (DAD) coupled to UPLC system: 211 nm for carbamazepine, 284 nm for *cis*-resveratrol and 306 nm for *trans*-resveratrol. Using these chromatographic conditions, the retention for trans-resveratrol and carbamazepine were 2.7 (figure 4) and 3.9 minutes, respectively.

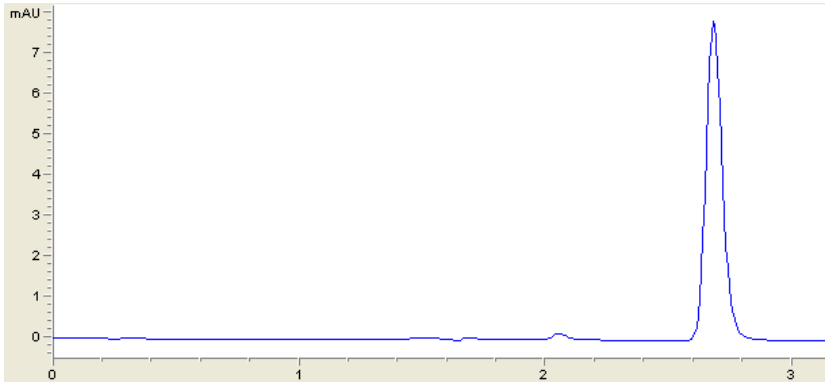


Figure 4. HPLC-DAD chromatogram of a 1 µg/mL resveratrol sample at 306 nm.

This method was validated according to the Food and Drug Administration (FDA) [82] and International Conference on Harmonisation (ICH) guidelines for validation of bioanalytical methods [83]. In this way, the method has been tested in terms of linearity, accuracy and precision. The liquid-liquid extraction procedure efficiency was also evaluated.

2.2.3.1. Method validation

Calibration Curve

The calibration curve had seven points equally distributed points ranging from 0.1 to 10 µg/mL. M9 media (Appendix 1) was used as blank sample in validation. M9 medium acidified with 50 µL of HCl 1M was spiked with *trans*-resveratrol to achieve the following final concentrations: 0.1, 0.25, 0.5, 2.5, 5, 7.5 and 10 µg/mL. This mixture was further spiked with 50 µL of carbamazepine (100 µg/mL). The extraction procedure was the same as described above and was performed for each sample.

Intermediate Precision

In intermediate precision, three points of different concentrations (2, 4 and 8 µg/mL) were tested in triplicate for five consecutive days. The procedure was similar to described below.

Intraday Precision

Intraday precision was measured with four distinct concentrations, encompassing the highest and the lowest concentrations, as well as two intermediate ones: 0.1, 0.5, 5 and 10 µg/mL. These extractions were performed in quintuplicate in only one day. The protocol was the same as already described.

Extraction Efficiency

Extraction efficiency was measured by the following equation:

$$\%\, Extraction\ Effiency = \frac{Real\ concentration\ (after\ extraction)}{Theoretical\ concentration\ (before\ extraction)} \times 100 \qquad (3)$$

Thus, two variations of the same protocol are used. In one of them, where resveratrol was added at the beginning, the extraction was carried out with resveratrol – real concentration. In the other, resveratrol was added only to the organic phase before drying, so, extraction was performed without resveratrol - theoretical concentration. The internal standard was added to both before dryness. The remainder protocol is the same described.

2.3. Screening assays

In order to assess resveratrol production in the two recombinant microorganisms and conclude which factors have more influence on production, shake flask assays were performed for the evaluation of six factors: *p*-coumaric acid concentration, optical density (OD_{600}) at precursor addition (induction experiments), growth temperature, agitation, medium composition and pH (only for *E. coli*) (Table 7). Each of these factors had several levels which were assessed to obtain a greater understanding about culture conditions of *E. coli* and *S. cerevisiae*.

Fermentations and pre-fermentations were carried out in 250 mL shake flasks with 62.5 mL of medium. Pre-fermentations grown up to a final OD_{600} of 2.6 and a dilution was performed to an initial OD_{600} of 0.05 in fermentations.

The background conditions were maintained, unless a particular factor was being tested. Thus, for *E. coli*, fermentations were carried out on M9 media and resveratrol production was induced at an OD_{600} of 0.1 with 1 mM of *p*-coumaric acid (Sigma-Aldrich, USA) dissolved in dimethyl sulfoxide (DMSO, purchased from VWR, USA). The fermentations were grown at 30 ºC, 250 rpm for 48 hours. Glycerol was used in M9 medium, since previous studies showed that glucose reduces STS expression and consequently resveratrol production [51]. In case of *S. cerevisiae*, fermentations were performed using SC media supplemented with tryptophan (Fluka, USA), for selection purposes, and it should be induced at same conditions than bacteria. The fermentations were grown at 30 ºC, 250 rpm for 100 hours and the screening assays are presented in table 9. Every four hours, samples were taken from each fermentation to evaluate resveratrol production by HPLC-DAD.

Table 7. Culture factors and ranges tested in Escherichia coli screening assays.

Factors	Range
p-coumaric acid concentrations (mM)	0; 1; 5; 10; 15 and 20
Optical density (OD_{600}) at time of induction	0.1; 0.2; 0.5; 1
Growth temperature (ºC)	25; 30; 37; 42
Agitation (rpm)	150; 200; 250; 300
Medium composition	Table 8
pH	5; 6; 7; 8; 9

Table 8. Medium composition tested for *E. coli* screening assays. In 'M9 base' and 'Glycerol assays', the concentrations of the presented compounds were modified in M9 base recipe, maintaining the remaining nutrients.

M9 base	M9 without yeast extract	Glycerol assays
NH_4Cl, 5 g/L Yeast extract, 5 g/L	NH_4Cl, 5 g/L NH_4Cl, 5 g/L Triptona, 2.5 g/L Triptona, 5 g/L	Glycerol, 20 g/L Glycerol, 2.5 g/L Glycerol, 2 g/L Glycerol 1.5 g/L

Table 9. Ranges and culture factors tested in Saccharomyces cerevisiae screening assays.

Factors	Variables
p-coumaric acid concentrations (mM)	0; 2; 5; 10; 15 and 20
Optical density (OD_{600}) at time of induction	0.1; 0.2; 0.5; 1
Growth temperature (ºC)	25; 30; 37; 42
Agitation (rpm)	150; 200; 250; 300
Medium composition	Tryptone, 5 g/L; yeast extract, 5 g/L; peptone, 5 g/L

All the media and solutions were sterilized (Uniclave 88, AJC, Portugal) and techniques were performed in aseptic conditions. Once the screening assays were performed, the data obtained regarding the most relevant factors and their ranges were used to optimize resveratrol production using Design of Experiments.

2.4. Design of Experiments

In order to generate data for bioreactor assays, Design Expert® Version 7.0.0 (from Stat-Ease Inc., USA) was used. The initial design chosen was Central Composite Rotatable Design (CCRD), with no blocks. Once we have 4 factors (k) and taking into account what was described in section 1.4.1, the total number of runs are 30 $(2^4 + (2 \times 4) + 6)$. In order to obtain the data for bioreactor operation, the range of values of each of four variables was defined as follows, based on data obtained in screening assays:

- Precursor concentration: 0 – 16 mM
- Optical Density (OD_{600}) at time of induction: 0.125 – 1.025
- pH: 6 – 8
- Temperature (ºC): 25 – 37

These values were chosen taking into account the maximum value of the production at each factor tested as well as neighboring values, in order to cover a greater range of values which contains all the probable values for an optimal resveratrol production. Applying to coded levels as presented in table 4, the values of the codes were calculated as shown in table below (table 10):

Table 10. Independent variables and CCRD levels used to determine the variables levels of 30 assays.

Variable	Coded variable level				
	Lowest $-\beta$	Low -1	Center 0	High $+1$	Highest $+\beta$
Precursor Concentration (mM)	0	4	8	12	16
OD_{600} induction	0.125	0.35	0.575	0.8	1.025
pH	6	6.5	7	7.5	8
Temperature (ºC)	25	28	31	34	37

The test table generated was as follows (table 11):

Table 11. Matrix generated with the assays, the four factors and several levels studied. The factors are precursor concentration (mM), optical density at precursor addition, pH and temperature (ºC).

Run	Precursor concentration (mM)	OD_{600} at precursor addition	pH	Temperature (ºC)
1	0	0.575	7	31
2	4	0.8	7.5	28
3	12	0.8	6.5	28
4	12	0.35	6.5	34
5	12	0.35	7.5	28
6	8	0.575	7	31
7	8	0.575	6	31
8	4	0.8	6.5	28
9	4	0.35	6.5	28
10	4	0.8	7.5	34
11	8	0.575	8	31
12	12	0.8	7.5	34
13	8	0.575	7	31
14	4	0.8	6.5	34
15	4	0.35	7.5	28
16	4	0.35	6.5	34
17	8	0.575	7	31
18	12	0.35	7.5	34
19	12	0.8	6.5	34
20	8	0.575	7	31
21	8	0.575	7	31
22	16	0.575	7	31
23	8	1.025	7	31
24	8	0.575	7	25
25	8	0.575	7	37
26	8	0.125	7	31
27	4	0.35	7.5	34
28	8	0.575	7	31
29	12	0.8	7.5	28
30	12	0.35	6.5	28

The answer of the model (y) was termed 'resveratrol concentration' and it was a response surface study type. A detailed analysis of variance (ANOVA) was performed using the software indicated above.

After obtaining the grid trials, the scale-up was performed in bioreactors.

2.5. Bioreactor operation

Four 0.5 L working volume parallel bioreactor (Infors HT, Switzerland) with a double blade impeller were used to culture *E. coli* in 250 mL of M9 medium. Solutions and antibiotics were added using sterile syringes and needles through the inoculation port. Shake flask cultures were combined to inoculate the bioreactor at an initial OD_{600} of 0.05.

Bioreactor conditions were maintained at 250 rpm and 30 % of dissolved oxygen concentration, with variable temperature and pH. The dissolved oxygen level was controlled by manipulating the oxygen concentration in the gas stream. All the parameters were monitored continuously using IRIS software (Infors HT, Switzerland) and the cultures grew under subdued light in order to avoid *trans*-resveratrol isomerization. The pH was maintained through the automatic addition of 1 M NaOH (Fisher Scientific, USA) and 1 M H_2SO_4 (Panreac, Spain).

Fermentations were carried out for 30 hours and samples were taken aseptically at 22 and 30 hours of growth. These samples were used for resveratrol quantification, flow cytometry and real-time qPCR using an appropriate dilution.

2.6. Flow cytometry

BOX BOX (Invitrogen, USA) solution at 1 mg/mL was prepared in DMSO and stored at – 20 ºC. PI solution at 1 mg/mL in water (Sigma-Aldrich, USA) was stored at 4 ºC. Both were kept protected from light. Samples were analyzed on CyAn ADP (Beckman Coulter, USA) and acquisition was performed with software Summit 4.3 (Beckman Coulter, USA). Fluorescence and light-scatter signals resulted from a 20 mW semiconductor laser at 488 nm. Fluorescence signals were acquired logarithmically as well as light-scatter signals. FSC, SSC and fluorescence where accumulated and the fluorescence signal was screened by FL1 and FL3 bandpass filters. To reduce electronic and small particle noise threshold levels were set on SSC and data acquisition for a single sample took about 3 minutes and 5000 events were gathered.

The samples taken from fermentation were used for flow cytometric analysis using a PI/BOX dual staining. The samples were diluted in phosphate-buffered saline (PBS) buffer (pH 7.4) supplemented with 4 mM of Ethylenediamintetraacetic acid (EDTA), pH 7.4 to reach a final cell concentration of $1.0x10^6$ cells/mL. These cells were incubated with 1 µg/mL PI and 2.5 µg/mL BOX for 15 minutes in the dark, at room temperature. Afterwards, labeled cells were centrifuged at 5000 rpm for 5 minutes at room temperature, washed with PBS (pH 7.4) and

acquired in the flow cytometer. The fluorescence signals were collected by FL1 (BOX) and FL3 (PI) bandpass filters.

2.7. Real-time qPCR

2.7.1. Sample preparation

Samples were prepared collecting 1 mL of cell culture containing 1.0×10^8 cells that were heated in a 1.5 mL eppendorf for 10 minutes at 95 ºC, followed by an immediate storage at -20 ºC. One unit of OD_{600} correspond to 3.97×10^8 CFU/mL, and this correlation was used throughout this work to determine cell concentration in each sample.

2.7.2. Real-time qPCR

Quantitative real-time PCR was used for the absolute determination of PCN in *E. coli* cells. Specific primers (Stab Vida, Portugal) for chloramphenicol resistance gene (forward: 5'-ACCGTAACACGCCACATCTT-3'; reverse: 5'-TTCTTGCCCGCCTGATGAAT-3') and ampicillin resistance gene (forward: 5'-TCCTTGAGAGTTTTCGCCCC-3'; reverse: 5'-TTCATTCAGCTCCGGTTCCC-3') were used to amplify fragments in each of the two plasmids used. Real-time PCR efficiency was determined for primer set using standard solutions of known plasmid copy number (1:1, 1:10, 1:100; 1:1000; 1:10000). Real-time PCR (IQ5 Biorad, USA) reactions were performed using 3 µL of sample for a 20 µL reaction containing 10 µL of Maxima^{TM} SYBR Green qPCR Master Mix (Fermentas, Canada), 400 mM of pAC-4CL1 primer (forward and reverse) or 200 mM of pUC-STS primer (forward and reverse). For pUC-STS, reactions were incubated at 95 ºC for 3 minutes, followed by 30 cycles of 10 seconds at 95 ºC and 30 seconds at 58 ºC, resulting in a 102 % amplification efficiency. For pAC-4CL1, reactions were incubated at 95 ºC for 3 minutes, followed by 30 cycles of 10 seconds at 95 ºC and 30 seconds at 60 ºC, where the efficiency obtained was 109.5 %. Bacterial cell concentration was kept constant at 3×10^4 cells/reaction. PCN standards for calibration curve were made spiking purified plasmid DNA with non-transformed *E. coli* cells, according to a previously described method [81]. In this way, each reaction contains different amounts of plasmid DNA and 3×10^4 cells/reaction. PCN calculation is based on plasmid base pair number (3622 bp for pUC-STS and 6171 bp for pAC-4CL1). An average of three assays composes each point of the calibration curve. Acquisition and analysis were performed in BioRad IQ 5 Software.

Chapter 3

3. Results and Discussion

3.1. Transformation of *Escherichia coli* with pAC-4CL1 and pUC-STS

Escherichia coli transformation by thermal shock with the two plasmids was successfully achieved. After transformation, cells were spread in LB plates with ampicillin and chloramphenicol and after overnight growth several and separated young colonies had grown. The selective medium inhibited growth of any other species, once colonies were morphologically similar (round and light yellow). The presence of colonies meant that bacteria had plasmids pAC-4CL1 and pUC-STS inside, which was further confirmed after plasmid extraction using a digestion procedure with Hind III and subsequent agarose gel electrophoresis (Figure 5). Here, the first band corresponds to plasmid pAC-4CL1 which has 6171 bp, and the second band corresponds to pUC-STS, which have 3622 bp.

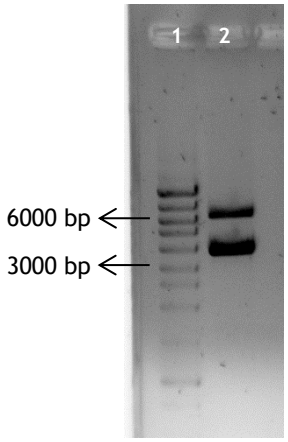


Figure 5. Agarose gel electrophoresis of digested plasmid DNA obtained from transformed *E. coli* with two plasmids (lane 2) and molecular weight marker (lane 1).

3.2. Method validation

3.2.1. Optimization of extraction procedure

Culture media samples contained resveratrol along with several other compounds, making it a complex matrix which led to the development of an extraction procedure to guarantee adequate sample purification and consequently enhance the chromatographic column lifetime and performance [17].

This extraction procedure was optimized and chosen between three procedures tested. The first protocol (hereinafter as 'procedure 1') was not very different from the used protocol. 'Procedure 2' and 'procedure 3' were studied in previous works (not published) and tested in this work in order to choose the best extraction method. The next figure (figure 6) is a scheme of the procedures used in this study.

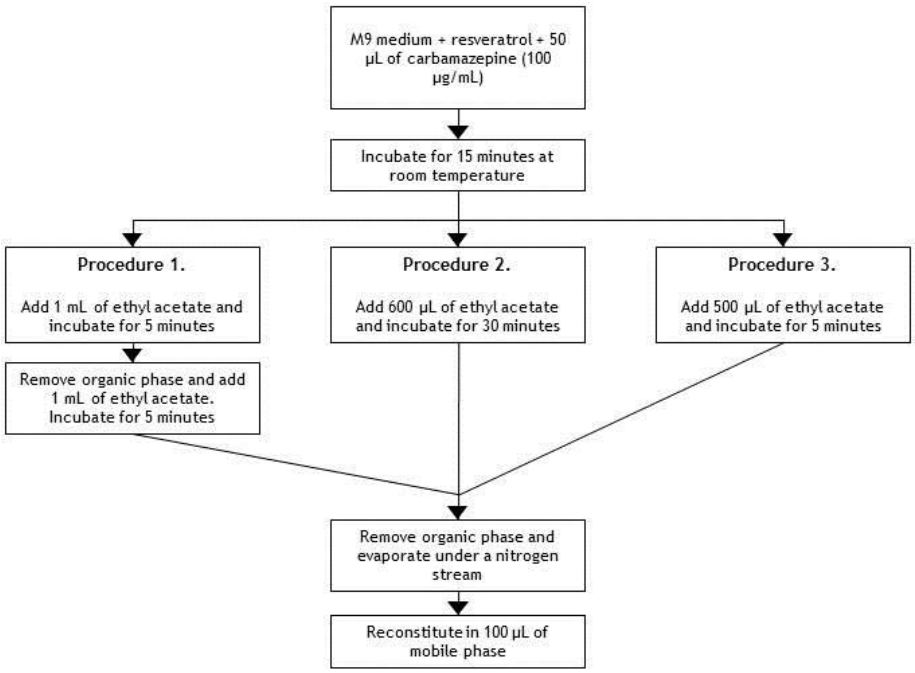


Figure 6. Schematic representation of the three different procedures tested.

Medium samples spiked with resveratrol were used in all these procedures. Extractions in blank samples and resveratrol samples were performed according to the three protocols tested. These assays were carried out using YPD and M9 media (Appendix 1). After extraction with all three protocols, In YPD medium, an interferent peak was observed on the blank sample with approximately the same retention time as resveratrol. In this way, we have chosen to perform *S. cerevisiae* cultures in SC media in every stages of the work involving yeast since, for this medium, no interfering substance could be seen at resveratrol retention time. In order to assess which method was the best, a sample injection of 10 µg/mL was performed after an extraction according to procedure 1, already referenced [51]. It was obtained a peak of 36.6 mAU x min and was taken as reference. Injections with the same concentration of resveratrol were made according to the three procedures and the results were compared to the previous peak (table 12).

Table 12. Results obtained from the three different procedures.

Area (mAU x min)	Procedure 1	Procedure 2	Procedure 3
Resveratrol	32.3	24.1	19.8
Carbamazepine (100 μg/mL)	2814.4	2891.4	2799.5

After analyzing the results (table 12), it can be concluded that the nearest value (comparing to the obtained previously) was 32.3 and was achieved using procedure 1. For this reason, procedure 1 was chosen and used as extraction protocol for samples from *E. coli* and *S. cerevisiae* fermentations. However, this procedure was changed and simplified. This protocol was performed with only one recovery of organic phase, carried out after the first 5 minutes of incubation and then the sample was injected, obtaining an area of 37.4 mAU x min. Comparing with the value obtained with two extractions (36.6 mAU x min), the difference was not significant and, hereinafter, this procedure was performed with only one ethyl acetate recovery. SC and M9 media proved to be suitable for both cultivation and resveratrol quantification, since they do not present any important interfering substance in the chromatograms. In other studies for resveratrol production [17].

3.2.2. Calibration curve and precision

In order to determine the linearity of the method, a calibration curve was performed at concentrations ranging from 0.1 to 10 μg/mL. Seven calibrators were prepared in simulated samples and analyzed using the procedure described. The calibration curves were obtained by plotting the area ratio between resveratrol and IS versus resveratrol concentration and the results were submitted to a linear regression (table 13). Accuracy (mean relative error – bias – between measured and spiked concentrations) was far below ±15% for all concentration levels [82, 83]. The middle values on calibration curves may be problematic, and sometimes the calibration range has to be divided in two calibration curves in order to maintain the linearity [17]. In this case, it was not necessary to split in half the calibration curve and linearity was achieved with acceptable values [82, 83], which constitutes and advantage of the developed method. However, this values were achieved after submit the ratio between resveratrol and IS to a ponderation, and the factor used was the inverse square of the ratio, as presented in table 13.

Table 13. Linearity parameters (n=5). When applicable, values are presented as mean values ± standard deviation.

Ponderation	Linearity (ug/mL)	Slope ± SD	Intercept ± SD	R^2 ± SD
$\frac{1}{x^2}$	0.1 – 10	0.17±0.01	-0.01 ± 0.00	1.00 ± 0.00

Intraday precision measures the method repeatability and is performed a set of assays always under the exact same conditions in a short interval of time, generally one day [84]. Four concentrations were tested and they ranged from 0.1 to 10 μg/mL, as can be seen in table 14.

Table 14. Intraday precision data table (n=5). When applicable, values are presented as mean values ± standard deviation.

Resveratrol Spiked (μg/mL)	Mean ± SD	CV (%)[1]	RE (%)[2]
0.1	0.10 ± 0.00	2.13	-4.95
0.5	0.47 ± 0.01	1.79	-7.40
5	5.35 ± 0.13	2.46	6.55
10	10.93 ± 0.22	2.05	8.51

The closeness of mean test results obtained by the method to the true concentration of the analyte is the accuracy of an analytical method. In relation to the closeness of individual measure of an analyte when the procedure is applied to multiple aliquots of a single homogeneous volume of biological matrix, it is precision [82, 83]. Accuracy and precision were evaluated by interday precision. Accuracy is the difference between the concentration of the analyte, which is known, and the concentration obtained by the method. In this parameter were evaluated the same 7 points of the calibration curve (table 15).

Table 15. Interday precision results (n=5). When applicable, values are presented as mean values ± standard deviation.

Resveratrol Spiked (μg/mL)	Mean ± SD	CV (%)	RE (%)
0.1	0.10 ± 0.00	1.58	3.59
0.25	0.23 ± 0.01	2.65	-5.94
0.5	0.47 ± 0.03	6.43	-7.67
2.5	2.50 ± 0.10	4.02	-2.58
5	4.98 ± 0.18	3.56	-2.92
7.5	7.83 ± 0.31	4.00	4.85
10	10.37 ± 0.26	2.54	4.77

The last parameter evaluated was intermediate precision, which testes the method reproducibility, analyzing different concentrations in an extended time and in slightly

[1] CV (%) = Coefficient of Variation
[2] RE (%) = Relative Error (measured concentration-spiked concentration/spiked concentration) x 100

different conditions. Intermediate precision was evaluated in three concentrations as shown in table 16.

Table 16. Intermediate precision results (n=15). When applicable, values are presented as mean values ± standard deviation.

Resveratrol Spiked (µg/mL)	Mean ± SD	CV (%)	RE (%)
2	2.00 ± 0.17	8.71	-1.13
4	4.00 ± 0.25	6.15	0.97
8	8.26 ± 0.41	5.00	2.89

After analyzing the data obtained, it can be concluded that all these values are accepted within the guidelines for bioanalytical method validation [82, 83]. This method is reproducible, precise and accurate, because there is a small variation between the values analyzed and the values obtained, which is expressed by standard deviation (SD). Nevertheless, this method is also repeatable, because the associated error in intraday precision is small. Furthermore, this method is reliable to low concentrations, since the standard deviation associated is always very low. This is important in the sample quantification from *S. cerevisiae* fermentations, once the microorganism produces low levels of resveratrol.

3.2.3. Extraction efficiency

Extraction efficiency is the measurement of analyte recovery in given assay [82], being calculated as already described in equation (3), section 3.2.3.1. It was evaluated at three concentration levels corresponding to low (0.1 µg/mL), medium (2.5 µg/mL) and high (10 µg/mL) resveratrol levels with three replicates being performed for each concentration. Similar extraction efficiencies were found in other studies [17], both near to 100 % to maximal and minimal points. Although LLE is more cost-effective and versatile technique, a discrepancy in the values comparing between these two studies lies on 2.5 µg/mL concentration, where the valued obtained in our work were slightly lower. This calibration curve covers the range of concentration used and, in the majority of cases, the values achieved were closer to the maximum and minimum values of the curve. For this reason, extraction efficiencies obtained were acceptable and closer to the obtained in other studies [17]. The extraction efficiencies are exposed in table 17.

Table 17. Extraction efficiency results. When applicable, values are presented as mean values ± standard deviation.

Resveratrol Spiked (µg/mL)	Mean ± SD	CV (%)	RE (%)
0.1	102.00 ± 6.63	6.63	6.50
2.5	72.40 ± 8.87	8.87	12.26
10	93.18 ± 8.60	8.60	9.23

3.3. Screening assays

After a successful validation of the analytical method, several tests were performed in order to determine which culture conditions yielded the highest resveratrol concentrations and also which were the most relevant parameters to take into account when developing an experimental design approach. The strategy used in screening assays was a one-factor-at-a-time approach. This methodology consists in selecting a baseline set of levels, for each factor, and then, successively vary each factor over its range keeping the other factors constant [59]. So, five factors were tested for *S. cerevisiae* and *E. coli*: temperature, medium composition, agitation, optical density (OD_{600}) at time of precursor addition and precursor concentration. Since *E. coli* proved to be the most suitable recombinant microorganism for resveratrol production, in a later stage, the influence of the pH on resveratrol production was also evaluated but only in *E. coli*. These two microorganisms were cultivated in similar growth conditions in order to compare resveratrol production. Samples were taken every four hours until 48 hours for *E. coli* and 100 hours to *S. cerevisiae* to assess resveratrol production in culture media yields. Two figures (7 and 8) represent a typical chromatogram from sample fermentation and the carbamazepine peak at 211 nm, respectively.

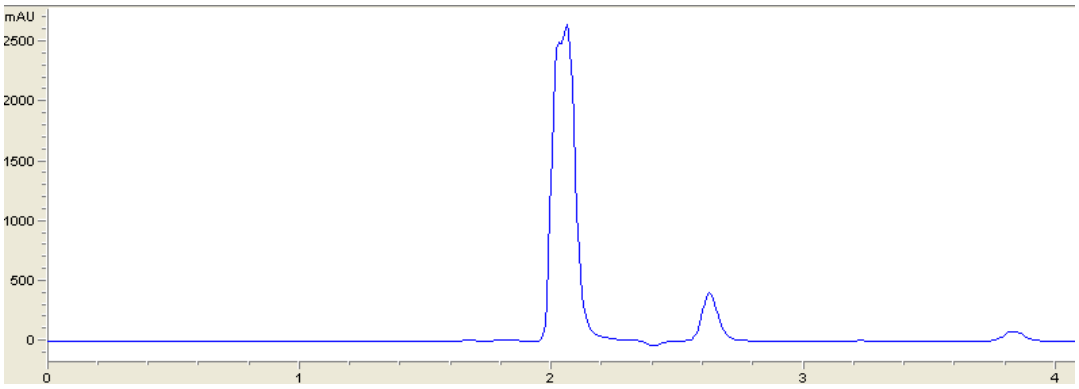


Figure 7. Typical HPLC-DAD chromatogram obtained from fermentation samples at 306 nm. The retention times for *p*-coumaric acid, resveratrol and carbamazepine were 2.01, 2.70 and 3.90 min, respectively.

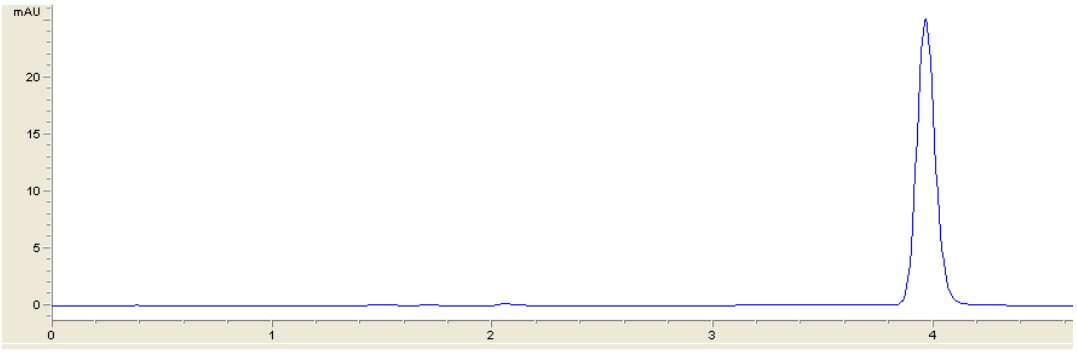


Figure 8. Typical HPLC-DAD chromatogram for carbamazepine (3.9 min) obtained at 211 nm.

Although some studies were already performed in order to evaluate the influence of the concentration of the precursor added [51] in resveratrol production by this recombinant microorganism, this study evaluates the influence of additional factors as temperature and pH, in order to allow a more comprehensive study about the influence of culture conditions in resveratrol production by this *E coli* strain.

3.3.1. Precursor concentration

p-Coumaric acid concentration was tested for six concentrations: 0, 1 or 2, 5, 10, 15 and 20 mM. This concentration range was chosen based on previous experiments [51]. The goal of this assay was the evaluation of the best precursor concentration to produce resveratrol.

In table 18, at 0 mM, a very small peak was detected in both microorganisms, which is considered as null. It was expected that, at 0 mM, resveratrol production results were zero, as the precursor was not added. Regarding *E. coli*, the best *p*-coumaric acid concentrations were 1, 5 and 10 mM, the latter being the one that yielded the higher resveratrol production (table 18). The results obtained in this work for *E. coli* are in agreement with previous studies that reported a resveratrol yield of about 104.5 ± 4.4 μg/mL, but in our case, a markedly higher concentration of precursor was added [51].

Above a precursor concentration of 15 mM, production starts to decrease, as well as OD_{600} (table 18). Decreasing growth may be associated with higher presence of DMSO in cellular culture, once *p*-coumaric acid is dissolved in DMSO and DMSO has a toxic effect to cells [85]. If the concentration of precursor increases, also increases DMSO concentration. In this way, cells do not grow as usually and resveratrol is not produced (table 18).

Table 18. Influence of precursor concentration on resveratrol production by E. coli and S. cerevisiae.

Precursor concentration (mM)	*Escherichia coli*		*Saccharomyces cerevisiae*	
	Max production (µg/mL)	OD_{600}	Max production (µg/mL)	OD_{600}
0	1.05	0.74	0.52	4.52
1 (bacteria) 2 (yeast)	100.24	4.47	2.55	5,16
5	91.03	4.24	2.96	2.26
10	105.65	3.10	---	---
15	72.15	2.03	---	---
20	53.15	2.60	---	---

It was observed in all experiments that resveratrol production has its maximum at the stationary phase (figure 9).

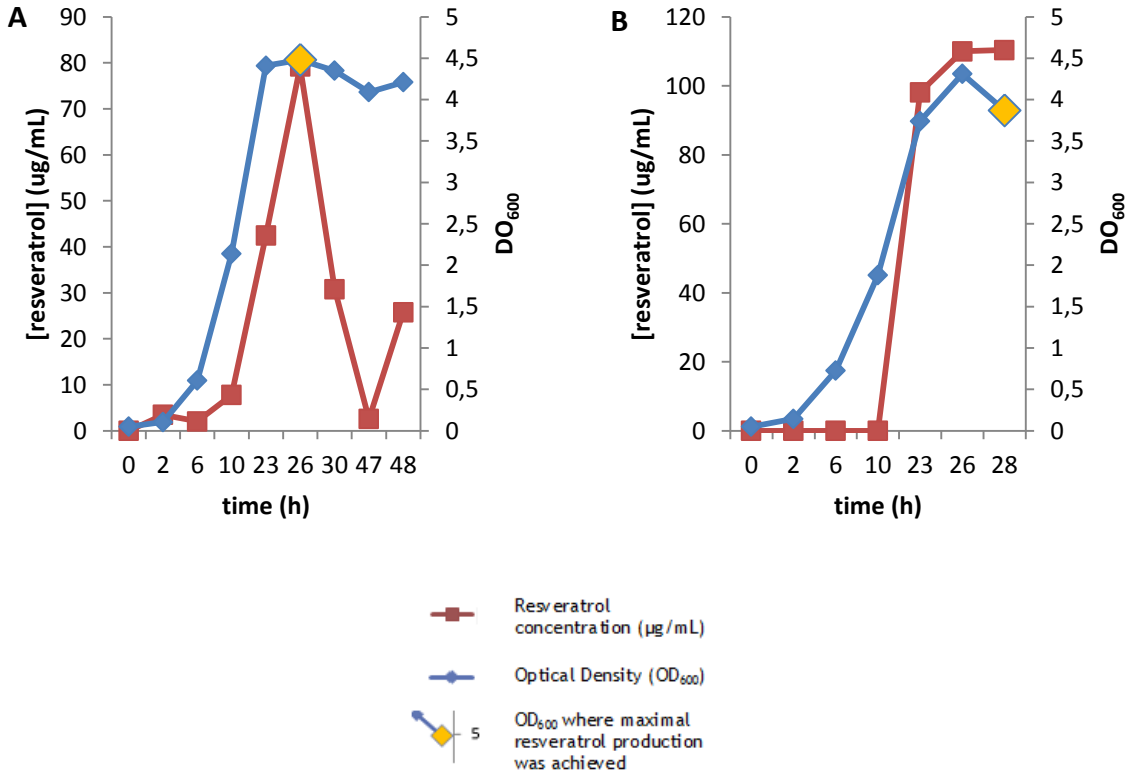


Figure 9. Variations on resveratrol production and optical density at 600 nm during shake flask fermentations with (A) 25 ºC and (B) pH 7.

As seen in figure 9, the maximum of resveratrol production is achieved at the beginning of stationary phase (figure 9A, assay 25 ºC, 26 hours) or even in the middle of stationary phase (figure 9B, assay pH of 7, 28 hours). Another study [51] presents a similar growth and production as achieved in figure 9A; however, in our case, generally the maximum of production was achieved later, as in figure 9B. That is the reason of starting sampling only

after 20 hours of fermentation and to stop fermentation 28 hours later. Based on comparison assay described on section 3.3 (and present in this set), at 1 mM of precursor, the OD_{600} is always higher than 3.5 (table 18).

Similarly, in *S. cerevisiae*, although growth was always present, even at higher concentrations (not shown) resveratrol was not produced at concentrations above 5 mM, where the maximum of production was attained. The motive for no accumulation of resveratrol at high *p*-coumaric concentrations is not known and the reasons could be similar to those presented for *E. coli*, because *S. cerevisiae* is also susceptible to high concentrations of DMSO. However, it might be ascribed to the fact that phenolic acids such as ferulic acid, levulinic acid and *p*-coumaric acid are able to interrupt central metabolism in the yeast cell [15], leading to a diminished cell growth as was seen by the decreasing OD_{600} while precursor concentration increases.

3.3.2. Optical density (OD_{600}) at time of precursor addition

Optical density (OD_{600}) at the time of precursor addition was another tested parameter and results are shown in table 19. This assay involves four levels as described below. In these tests, the aim was verify if optical density interferes with the final resveratrol production and assess which stage of growth was more advantageous for the addition of *p*-coumaric acid.

In relation to *E. coli*, optical densities above 0.2 seemed to influence the final amount of resveratrol. The assay performed at an OD_{600} of 0.1 (comparison assay) had a lower concentration than expected, which may demonstrate that other optical densities may be more effective. In this way, the highest resveratrol concentration (104.68 µg/mL) was obtained at OD_{600} of 1, which means that the addition of precursor in the early stages of growth affects *E. coli* growth as *p*-coumaric acid increases the lag phase due to damage caused to cells [86] and consequently affects resveratrol production.

Nevertheless, growth was slightly affected as the optical density at time of induction increased. However, this event does not have effect on resveratrol production, which could result in higher specific yields. On the contrary, optical density in *S. cerevisiae* assays remained relatively constant. The production corresponding to an OD_{600} of 0.1 was the maximum obtained, which do not corresponds to maximum OD_{600} achieved (5.42, table 19). Therefore, it may be concluded that the optical density at the time of addition of precursor does not have a great effect in resveratrol production by *S. cerevisiae*. However, it may be probable that low optical densities favor resveratrol production in this yeast.

Table 19. Influence of optical density (OD600) at time of addition of precursor on resveratrol production and microbial growth for both microorganisms.

Optical density (OD_{600})	*Escherichia coli*		*Saccharomyces cerevisiae*	
	Max production (µg/mL)	OD_{600}	Max production (µg/mL)	OD_{600}
0.1	91.76	6.19	3.17	4.86
0.2	101.73	6.14	0.65	4.47
0.5	102.74	4.59	1.18	4.76
1	104.58	3.98	1.33	5.42

3.3.3. Temperature (ºC)

Temperature is one of the key factors that control the growth of all microorganisms – and probably is the most important, at either too cold or too hot a temperature, microorganisms will not be able to grow and may even die [50]. The minimum and maximum temperatures for growth vary greatly among different microorganisms and in extreme temperatures, microorganisms will not be able to grow [50]. In this way, temperature assays were performed in four levels, (25, 30, 37 and 42 ºC). Both microorganisms are mesophiles (with midrange temperature optima, from 25 to 40 ºC) [50].

Observing the results presented in table 20, it can be concluded that yeasts grow better at lower temperatures while bacteria grow better at higher temperatures, which is in accordance with literature generally suggests 30 ºC [18] as optimal growth temperature to *S. cerevisiae* and 37 ºC for *E. coli* [50]. As the temperature rised, *E. coli* achieved higher optical densities at the beginning of stationary phase. However, these optical densities might not correspond to a real growth, but to cell filamentation, because high-copy number plasmid (as pUC-STS contained in *E. coli*) maintenance and replication imposes a metabolic burden on the cell, resulting in downregulation of cell wall biosynthetic genes [87], leading to increased cell size. This hypothesis in emphasized by the fact that at 37 and 42 ºC resveratrol production was low (table 20). The decrease in production can also be explained by possible resveratrol degradation at temperatures above 30 ºC, as demonstrated in a study [54] which submitted resveratrol under heat treatment, resulting in a decreased content of resveratrol under temperature stress (35 ºC).

In *E. coli*, 30 ºC was the temperature which yielded the higher resveratrol amounts. For *S. cerevisiae*, 42 ºC is a not recommended temperature, neither for growth nor resveratrol production, since a very slightly growth was detected (data not shown) and resveratrol was not quantified in any samples at any hour. Observing the optical density, *S. cerevisiae* is apparently more sensitive to temperature than *E. coli* (table 20). This affirmation is justified by the fact that there is a noticeable difference between production at 25 ºC and at 30 ºC. However, between 30 and 37 ºC there is not a perceptible difference (table 20).

Nevertheless, the best temperature for resveratrol production in *S. cerevisiae* was 30 ºC, the optimal temperature for fermentation for this yeast and also protects resveratrol from degradation (the literature about resveratrol production by *S. cerevisiae* describes 30 ºC as the best temperature).

Although the optimal growth temperature for *E. coli* is about 37 ºC, as seen in some preinoculums [16], growth temperatures during production of resveratrol were 30 ºC [51] or lower (28 ºC, [18]). Bacteria in preinoculum can growth at its optimal temperature, because it will be diluted before start fermentation. During fermentation, *E. coli* still can grow at 37 ºC; however, when precursor or expression inducer is added (as IPTG), the fermentation is placed immediately at 30 ºC [16], to protect resveratrol and the compound added. Other studies [18], [51], performed fermentations always at same temperature, probably to protect resveratrol and cells from a heat shock.

Table 20. Effect of temperature (ºC) on resveratrol production and optical density in E. coli and S. cerevisiae fermentations.

Temperature (ºC)	*Escherichia coli*		*Saccharomyces cerevisiae*	
	Max production (µg/mL)	OD_{600}	Max production (µg/mL)	OD_{600}
25	66.53	3.94	0.60	1.69
30	83.10	4.48	1.23	5.02
37	19.95	5.24	1.21	4.18
42	12.74	5.69	---	---

3.3.4. Agitation

Agitation is a growth condition discussed in several production studies [58]. It is commonly associated with aeration but also with the production of foam, a potential toxic environmental condition which interferes with oxygen transfer rates due to a large contact surface [88, 89], thus influencing cell growth and ultimately, process productivity. To solve this problem, several anti-foam molecules, mostly organic molecules, were developed and are sometimes added to fermentations in order to control or stop foam production. However, these compounds can also be toxic to cells and decrease oxygen concentration. In this assay, several agitations were tested in order to determine the best for resveratrol production in *E. coli* and *S. cerevisiae*.

In *E.coli,* apart from 150 rpm, there was no great difference in agitation speeds ranging from 200 to 300 rpm as can be seen in table 21. In this microorganism, although the optical density was higher at 300 rpm, resveratrol production was not. The best agitation speed for resveratrol production was 250 rpm, in the case of *E. coli* (105.25 µg/mL, table 21). Besides this detrimental effect in production, at 300 rpm, an increased production of foam was also observed and, therefore, one can conclude that this speed was not suitable for this

production process. For *S. cerevisiae*, 200 rpm is the best option (table 21), due to the fact that the maximum production was achieved at this stirring speed.

At 300 rpm, this agitation speed may cause cellular disruption and decreasing of optical density, due to greater mechanical forces and shear stress [57]. Higher agitation rates may also vary the efficiency of growth and the rate of formation of the desired product can be seriously affected [89]. In addition, this was not an important factor in final production, as there were no differences in the range of speeds tested.

Table 21. Impact of agitation (rpm) on biomass and resveratrol production by E. coli and S. cerevisiae.

Agitation (rpm)	*Escherichia coli*		*Saccharomyces cerevisiae*	
	Max production (µg/mL)	OD_{600}	Max production (µg/mL)	OD_{600}
150	51.36	2.99	0.55	4.31
200	92.20	3.89	1.02	4.16
250	105.25	3.53	0.84	7.04
300	87.57	4.66	0.43	4.20

3.3.5. Medium composition

For a successful culture of any microorganism, growth conditions and medium should be suitable. In order to determine which medium composition would be the best to produce resveratrol, several assays were performed in *E. coli* and *S. cerevisiae*.

4.3.5.1. Medium composition assay for *Escherichia coli*

In this set of assays, media with different compositions were tested. Two nitrogen sources (NH_4Cl, yeast extract) were tested alone and combined at different concentrations and several concentrations of the carbon (glycerol) source were also evaluated.

As seen by the results in table 22, changing the composition of M9 media does not lead to an improved production, once all the results remain below 100 µg/mL [51]. NH_4Cl was increased five times relative to original M9 recipe (Appendix 1), yeast extract increased four times, as well as glycerol. However, a higher nutrient concentration in medium only seems to favor cell growth (in the case of yeast extract). The original recipe of this medium do not contains great amounts of carbon and/or nitrogen sources, which explains the low optical densities. Even if some of these sources are increased, is not sufficient to favor growth or resveratrol production.

Table 22. Influence of medium composition on biomass and resveratrol production by Escherichia coli.

Medium composition	Max production (µg/mL)	OD_{600}
M9 base + NH_4Cl (5 g/L)	93.46	3.82
M9 base + yeast extract (5 g/L)	76.81	7.67
M9 base + glycerol (20 g/L)	98.77	3.99
M9 base + NH_4Cl (2.5 g/l) – yeast extract	2.76	0.2
M9 base + NH_4Cl (5 g/L) – yeast extract	3.03	0.18
M9 base + tryptone (2.5 g/L) – yeast extract	40.88	4.58
M9 base + tryptone (5 g/L) – yeast extract	63.17	5.73
Glycerol (2.5 g/L)	70.35	4.32
Glycerol (2 g/L)	31.03	3.35
Glycerol (1.5 g/L)	28.43	3.67

4.3.5.2. Medium composition assay for *Saccharomyces cerevisiae*

These tests were performed by adding nitrogen sources (tryptone, yeast extract and peptone) to SC media, which contained glucose (20 g/L) as carbon source.

Similarly to *E. coli*, *S. cerevisiae* also used yeast extract to improve its growth (table 23). This increase was noticed in tryptone and peptone tests, but to a lower extent. Despite the increased cell growth, resveratrol production remained low. From all the tests presented here, yeast extract was the one which favor resveratrol production (table 23).

Table 23. Effects of medium composition on biomass and resveratrol production in Saccharomyces cerevisiae.

Medium composition	Max production (µg/mL)	OD_{600}
SC base + tryptone (5 g/L)	0.32	5.7
SC base + yeast extract (5 g/L)	1.76	13.44
SC base + peptone (5 g/L)	0.38	6.65

After performing all these tests, it was concluded that *S. cerevisiae* produces about 30 times less resveratrol than *E. coli*. The major advantage of *S. cerevisiae* over *E. coli* is its food-grade status, which may facilitate resveratrol production for applications in human nutrition, if incorporated in functional food or nutraceuticals [18]. However, in most trials performed in this study, yeast produced more resveratrol than in previous works [6, 15, 52], using similar growth conditions. Another work [18] has reported the use of 2 % galactose to induce gene expression, resulting in the highest resveratrol yields described for *S. cerevisiae* in the

literature. Nevertheless, the main goal of this study is produce resveratrol in higher amounts and, therefore, *E. coli* was chosen over *S. cerevisiae* for posterior resveratrol production optimization.

3.3.6. pH

pH is another key factor that influences microbial growth. *E. coli* is a neutrophile, because it grows optimally at a pH value in the range between 5.5 and 7.9 [50]. In this set of assays, pH was tested for five different values, and the pH at the end of fermentation was also measured to ensure that the pH variation obtained during cultivation did not influence resveratrol production and also to verify if the medium used could effectively maintain the desired pH.

Analyzing the data presented in table 24, the value of 7.0 allowed the obtention of the highest resveratrol yield (115.73 µg/mL). Apparently, pH does not affect cellular growth as the optical density remains around 4. The final pH was measured, and pH dropped about 0.49 to 1.85 from the starter values. This decrease of pH values may be explained by the normal metabolism of the cells. During fermentation, cells produce metabolites as acetic acid [78] which is released to culture medium, dropping its pH.

Although resveratrol is stable in a wide pH range [12] at pH values of 5 and 6, *E. coli* may have its growth affected at low pH, because an acidic medium may affect the expression of compounds, repressing or inducing the compounds and leading to acid tolerance, which influences growth and consequently, production [90]. Although resveratrol is stable, the acid tolerance of *E. coli* appears to be strong dependent on growth phase – stationary-phase or starved cultures show high levels of acid tolerance [90]. In the other hand, resveratrol is also affected by high pH values, as shown in a study [55], which describes the deprotonating of resveratrol at pH of 9. It can be concluded that the stability of resveratrol also depends on a matrix. It can be concluded that, being an important factor in cellular growth, pH also has repercussion in resveratrol production.

Table 24. pH influence in E. coli production of resveratrol.

pH	Max production (µg/mL)	OD_{600}	Final pH
5	67.54	3.49	4.5
6	60.31	4.06	4.93
7	115.73	3.87	6.51
8	77.65	4.32	7.06
9	83.13	4.19	7.15

The results obtained in these screening assays were already described to this strain of *E. coli* using lower concentrations of *p*-coumaric acid [51]. In literature, some articles describe greater amounts of resveratrol produced by *E. coli*, as 171 mg/L [9], but is more common to

find lower amounts [9, 18], as 3.6 mg/L. Another study describes great amounts of resveratrol, but after use inductors and specific expression constructs [16]. There is no report in literature of a detailed study about screening conditions of *E. coli* to produce resveratrol. However, a study was performed in similar conditions, using M9 medium with glycerol at 30 ºC for 48 h [51]. In this case, 104.5 µg/mL of resveratrol were achieved after about 20 hours of fermentation, at the beginning of stationary phase. As described in this study, resveratrol also appeared quickly after the addition of *p*-coumaric acid as in our case, but *E. coli* led about 22 - 24 h to achieve its maximal production. However, the same quantity was achieved, and even exceeded, in almost all screening assays. *p*-Coumaric acid interfered in cellular growth in similar ways in both studies. Also, in both cases, at high concentrations of precursor (mainly 20 mM), *p*-coumaric acid was not completely consumed and precipitated in the culture. However, this situation was noticed more often in *Saccharomyces cerevisiae*.

After performing these screening assays, the data recovered was used to optimize resveratrol production using a DoE approach. Later, a set of 30 assays was generated, and then carried out on bioreactor.

3.4. Design of Experiments

A four factor and five-coded level CCRD was used to determine the optimal resveratrol concentration. The four factors used were pH, temperature, precursor concentration and optical density at time of induction as the screening results suggested that these parameters had the greater influence on final resveratrol production. Six tests at center point were performed and a total of 30 experiments were required for this study. Considering the effects of main factors and the interactions between two-factor, as seen on table 4 and equation (1), where x_1 is precursor concentration (mM), x_2 is optical density (OD_{600}) at addition of precursor, x_3 is pH and x_4 is temperature (ºC), the equation of the model is [62]:

$$y = a_o + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 \quad (4)$$

The main effect (b_i) and two-factor interactions (b_{ij}) were estimated from the experimental data obtained in Design-Expert® Version 7.0.0.

After HPLC quantification at conditions described, the results were submitted in the referred software. Each point is the average of three injections. In the tables below (tables 25 - 28) are the data obtained after quantification in HPLC, the statistical parameters and equations obtained in Design Expert® Software. The model of analysis presented for resveratrol production was Polynomial Quadratic using a significance level of 0.05 % for the analysis.

Table 25. Statistical parameters obtained in Design Expert® (7.0.0) after an ANOVA analysis.

Parameters	22 hours	30 hours
F value	2.59	2.07
Prob > F (p-value)	0.0390	0.0879
Lack of fit F value	4.76	5.97
Lack of fit Prob > F (p-value)	0.0494	0.0311
R^2	0.7072	0.6584

Table 26. Statistical analysis ANOVA for the factors and interactions analyzed at 22 hours of fermentation. Legend: A = Precursor concentration (mM); B = OD600 at time of induction; C = pH; D = Temperature (ºC).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	12091.96	14.00	863.71	2.59	0.04
A	336.83	1.00	336.83	1.01	0.33
B	4.55	1.00	4.55	0.01	0.91
C	954.39	1.00	954.39	2.86	0.11
D	3533.97	1.00	3533.97	10.59	0.01
AB	27.25	1.00	27.25	0.08	0.78
AC	1440.04	1.00	1440.04	4.32	0.06
AD	149.29	1.00	149.29	0.45	0.51
BC	155.01	1.00	155.01	0.46	0.51
BD	86.87	1.00	86.87	0.26	0.62
CD	51.17	1.00	51.17	0.15	0.70
A^2	2424.68	1.00	2424.68	7.27	0.02
B^2	1021.54	1.00	1021.54	3.06	0.10
C^2	2615.27	1.00	2615.27	7.84	0.01
D^2	1415.05	1.00	1415.05	4.24	0.06
Residual	5005.50	15.00	333.70		
Total	17097.46	29.00			

Observing the data correspondent to 22 hours samples (table 26), the F-value presented makes the model significant because Prob > F value is lower than 0.050 (0.04). This implies that only 3.9 % chance (table 25) that F-value occurs due to noise. R-squared (or R^2) indicates how well the model fits the data in a line or curve. The R^2 (table 25) obtained for 22 hours was 0.7072, which means that 70.72 % of the response variability can be explained by this statistical model. p-value should be lower than 0.05 % to indicate that the model terms are significant. In the case of the factors studied at 22 h (table 26), only temperature was significant. The final equation in (terms of coded levels) estimated to resveratrol concentration response was:

$$y = 66.62 - 3.75x_1 + 0.44x_2 - 6.31x_3 - 12.13x_4 - 1.31x_2 + 9.49x_1x_3 + 3.05x_1x_4 - 3.11x_2x_3 + 2.33x_2x_4 + 1.79x_3x_4 - 9.40x_1^2 - 6.10x_2^2 - 9.76x_3^2 - 7.18x_4^2 \quad (5)$$

Table 27. Statistical analysis ANOVA for the factors and interactions analyzed at 30 hours of fermentation. Legend: A = Precursor concentration (mM); B = OD600 at time of induction; C = pH; D = Temperature (°C).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	34605.71	14.00	2471.84	2.07	0.09
A	451.65	1.00	451.65	0.38	0.55
B	214.71	1.00	214.71	0.18	0.68
C	1602.11	1.00	1602.11	1.34	0.27
D	14605.28	1.00	14605.28	12.20	0.00
AB	295.65	1.00	295.65	0.25	0.63
AC	3158.52	1.00	3158.52	2.64	0.13
AD	1511.80	1.00	1511.80	1.26	0.28
BC	176.17	1.00	176.17	0.15	0.71
BD	0.06	1.00	0.06	0.00	0.99
CD	391.07	1.00	391.07	0.33	0.58
A^2	3075.51	1.00	3075.51	2.57	0.13
B^2	1038.51	1.00	1038.51	0.87	0.37
C^2	8191.20	1.00	8191.20	6.84	0.02
D^2	3967.36	1.00	3967.36	3.31	0.09
Residual	17952.44	15.00	1196.83		
Total	52558.15	29.00			

Regarding the data correspondent to 30 hours samples (table 27), the F-value presented makes the model not significant, when considering a significance level of 0.05 %. There is an 8.79 % probability (table 25) that a model F-value (2.07) could occur due to noise. The R^2 (table 25) obtained for 30 hours was 0.6584, which means that 65.84 % of the response variability can be explained by this statistical model. If the fit of the method is not the best, the R^2 values will be affected. In this case, the p-value (table 27) of the factors studied which was lower than 0.05 % was temperature, as verified for the model concerning the 22h of fermentation. The final equation in (terms of coded levels) estimated to resveratrol concentration response was:

$$y = 101.80 - 4.34x_1 + 2.99x_2 - 8.17x_3 - 24.67x_4 - 4.30x_1x_2 + 14.05x_1x_3 + 9.72x_1x_4 - 3.32x_2x_3 - 0.064x_2x_4 + 4.94x_3x_4 - 10.59x_1^2 - 6.15x_2^2 - 17.28x_3^2 - 12.03x_4^2 \quad (6)$$

Both equations can be used to predict the response of the model when the containing factors vary. Another statistical and important term is lack of fit, which represents how suitable is

the model and this parameter should be not significant. The model of the samples from 22 and 30 hours have lack of fit of F-value significant (0.0494 and 0.0311, respectively).

After analyze the statistical parameters, it can be concluded that the model cannot be validated. However, some conclusions about optimization of resveratrol production were achieved.

Table 28. Results from CCRD performed in bioreactor. Legend: A (x_1) = precursor concentration (mM); B (x_2) = optical density (OD600) at time of induction; C (x_3) = pH; D (x_4) = temperature (°C); Mean Prod = mean of production; SD = standard deviation; CV = coefficient of variation. Samples were taken at 22 and 30 hours of fermentation.

Run	Variables				22 hours	30 hours
	A	B	C	D	Resveratrol concentration (µg/mL)	Resveratrol concentration (µg/mL)
1	0	0.575	7	31	1.53	3.11
2	4	0.8	7.5	28	41.71	97.56
3	12	0.8	6.5	28	39.38	64.72
4	12	0.35	6.5	34	9.32	13.41
5	12	0.35	7.5	28	40.00	79.32
6	8	0.575	7	31	65.17	83.49
7	8	0.575	6	31	28.66	32.53
8	4	0.8	6.5	28	86.30	159.96
9	4	0.35	6.5	28	84.10	153.73
10	4	0.8	7.5	34	21.17	20.18
11	8	0.575	8	31	22.21	26.32
12	12	0.8	7.5	34	16.13	15.77
13	8	0.575	7	31	61.52	124.56
14	4	0.8	6.5	34	58.52	61.99
15	4	0.35	7.5	28	39.48	65.54
16	4	0.35	6.5	34	32.37	35.06
17	8	0.575	7	31	65.09	90.20
18	12	0.35	7.5	34	11.69	14.90
19	12	0.8	6.5	34	17.51	15.90
20	8	0.575	7	31	80.67	119.47
21	8	0.575	7	31	74.34	100.59
22	16	0.575	7	31	52.24	109.28
23	8	1.025	7	31	23.71	78.01
24	8	0.575	7	25	57.94	69.17
25	8	0.575	7	37	13.59	31.72
26	8	0.125	7	31	56.45	69.87
27	4	0.35	7.5	34	13.81	23.22
28	8	0.575	7	31	52.90	92.50
29	12	0.8	7.5	28	28.57	50.68
30	12	0.35	6.5	28	23.51	46.07

As expected, if precursor concentration is 0mM, resveratrol production is null, since the values detected were lower than the limit of quantification for this method (0.1 µg/mL).

The highest amounts of resveratrol were usually obtained after 30 hours of fermentation (table 28). When 4 mM of precursor were added to the medium at pH of 6.5, 28 ºC and in an OD_{600} of 0.8, was obtained the highest production of resveratrol throughout the entire set of assays: 159.96 µg/mL (assay 8). It was also observed that low concentrations of resveratrol are generally associated with higher concentrations of *p*-coumaric acid. This relation can be seen in assays 8 and 3 (table 28), where 4mM of *p*-coumaric acid was added to assay 8 and 12 mM was added to assay 3. Both assays were performed only varying the precursor concentration and close to the conditions where the highest amount of resveratrol was achieved (28 ºC, at pH 6.5 and the precursor was added at an OD_{600} of 0.8), and, while assay 8 yielded 159.96 µg/mL of resveratrol, assay 3 allowed the achievement of almost half that value (64.72 µg/mL), with the increase of *p*-coumaric acid. Observing the response surface (figure 10) where the (B) optical density at time of precursor addition and (C) pH, it can be concluded that higher amounts of resveratrol are obtained if temperature and precursor concentration are minimal (28 ºC and 4 mM, respectively) when the optical density at time of precursor addition is at 0.80 and pH is at 6.5.

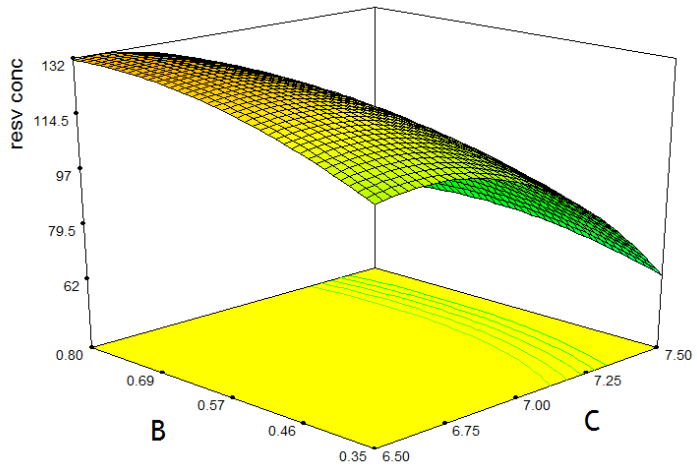


Figure 10. Response surface varying the (B) OD_{600} at the time of addition of precursor and (C) pH, at 30 h. The concentration of the precursor added and temperature were kept constant. 'Resv conc' is the response of the model (resveratrol concentration).

Analyzing the optical density at time of precursor addition, apparently the factorial points did not bring great variability to the final response. However, observing the assays 26 and 23, where were tested the axial points for this factor (OD_{600} of 0.125 and 1.025), it was not observed a great influence of this variation, resulting in an increase from 70 to 78 µg/mL of resveratrol with the raise of OD_{600} at time of precursor addition. Nevertheless, in the average of the six central points, 101.80 µg/mL were achieved. This highlights that at early stages of growth, cells are affected by the addition of *p*-coumaric acid, which could lead to an increase lag phase and cause a decrease in maximal growth rate [86], and consequently leading to a

decrease in resveratrol production. Furthermore, *p*-coumaric acid was dissolved in DMSO, a compound that can damage the cells due to its capacity to affect membrane permeability [77]. In the lag phase, the cells are biosynthesizing essential constituents [50], and the addition of *p*-coumaric acid and DMSO can interfere with growth rate and consequently, resveratrol production. In the exponential phase (OD_{600} of 1.025), growth also depends on culture conditions [50], and the addition of DMSO can cause a similar damage in the cells, slowing growth and resveratrol production.

Regarding to temperature, axial points as 25 ºC and 37 ºC, corresponding to assays 24 and 25 respectively, it was not yielded high resveratrol production, indicating that temperature also influenced the final production in bioreactor. The best results were obtained at 28 and 31 ºC, near to optimal temperature (30 ºC) obtained in screening assays. However, at low temperatures as 25 ºC (assay 24, table 28), *E. coli* did not produced high amounts of resveratrol, because 25 ºC is not the best optimal temperature for *E. coli* to growth, which leads to slower transport processes and growth [50], and consequently lower resveratrol production. Although 37 ºC is a temperature closer to the optimum growth temperature for *E. coli* [50], this temperature may led to resveratrol degradation [54], which resulted in lower production levels.

The influence of pH is slightly visible at axial point values (pH 6 and 8, assays 7 and 11, respectively. In assay 7 was obtained a yield of 32.53 µg/mL at pH 6, instead of 26.32 µg/mL at pH 8, in assay 11. This could indicate that lower pH values favored resveratrol production and also indicates that lower pH values favor the stability of resveratrol. Observing figure 11, maintaining constant optical density at time of precursor addition and temperature (0.575 and 34 ºC, respectively) it can be concluded that the lower amounts of resveratrol are obtained at higher values of pH.

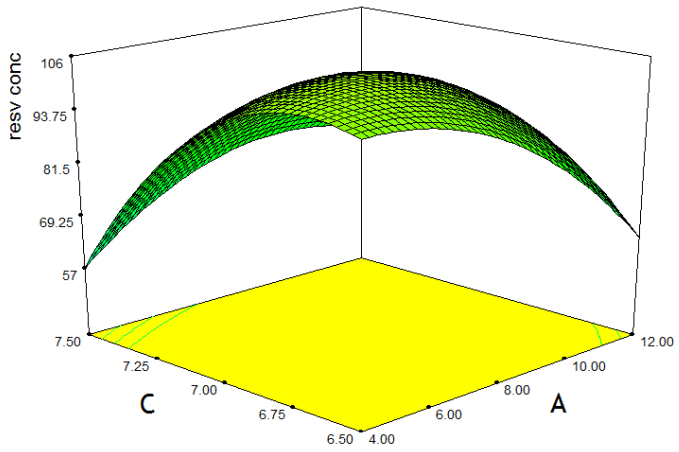


Figure 11. Response surface varying the (A) precursor concentration and (C) pH in relation to resveratrol concentration, at 30 h. 'Resv conc' is the response of the model (resveratrol concentration).

One of these assays performed in bioreactor allowed to achieve the highest amount of resveratrol for this strain of *E. coli*. This highlights the relevance of design of experiments,

because the conditions where the highest amount of resveratrol was obtained in screening assays, (addition of 10 mM of precursor at an OD_{600} of 1 to a culture grown at 30 ºC and pH 7) were different to those obtained after the optimization in bioreactor. The results demonstrated that addressing several factors at a time instead of a one-factor-at-a-time approach can provide better results due to the assessment of the simultaneous influence of various factors on overall production. It can be seen that the best resveratrol production yields were obtained at 28 ºC, pH 7, with a precursor concentration of 4 mM added at an OD_{600} of 0.8. However, one of the assays with the most similar conditions to those achieved in the screening assays (assay 21) still exhibited a value close to that obtained in the screening assays and in other study (100.59 µg/mL) [51], indicating that these assays were very reproducible.

So far, resveratrol production in bioreactor was performed only with plant cell systems [9], while resveratrol production using recombinant microorganisms has been carried out in a small scale shake-flasks [16]. As process productivity can be affected by plasmid segregational instability by the physiological states of cells [74], due to decrease plasmid and/or protein levels and cellular growth, these two parameters were monitored for each of these runs.

3.5. Cell physiology

In order to assess cell physiology, a dual-staining was performed using BOX and PI. BOX was used to evaluate the membrane potential, since only enters in depolarized cells and PI was used to verify the membrane integrity, as it only enters in the cell if it is injured. Following is presented a table (table 29) with the flow cytometry results obtained from 30 assays at two different hours.

Table 29. Effect of culture conditions on cellular viability evaluated by flow cytometry.

Run	PI (%)		BOX (%)	
	22h	30h	22h	30h
1	3.12	2.40	21.30	27.34
2	2.08	3.70	16.08	14.08
3	1.18	3.78	18.84	20.38
4	3.14	4.32	10.02	16.78
5	3.12	3.70	9.90	13.94
6	2.18	6.66	10.86	36.62
7	5.62	5.10	19.86	16.14
8	4.30	7.06	21.66	18.08
9	5.56	7.0	21.32	15.02
10	2.78	4.48	18.34	13.32
11	3.76	6.13	19.82	30.87
12	4.30	5.72	27.40	21.76
13	1.64	4.18	16.62	22.98
14	4.58	8.28	23.98	32.90
15	1.88	3.10	18.58	20.50
16	3.90	5.90	22.80	43.20
17	2.32	3.30	25	26.56
18	5.10	3.50	38.58	50.16
19	1.94	4.07	21.19	39.07
20	1.48	2.28	26.76	17.42
21	2.30	2.42	24.42	18.80
22	1.87	2.94	24.90	31.11
23	1.24	2.83	27.63	33.04
24	1.48	1.43	23.31	29.70
25	2.42	4.60	11.51	21.78
26	1.37	2.13	23.42	27.08
27	2.93	5.88	8.82	18.8
28	0.98	2.34	30.77	39.15
29	1.17	1.26	30.95	26.70
30	0.28	1.48	30.23	27.68

And here is presented a figure (figure 12) concerning the assays which yielded the highest and the lowest resveratrol production values.

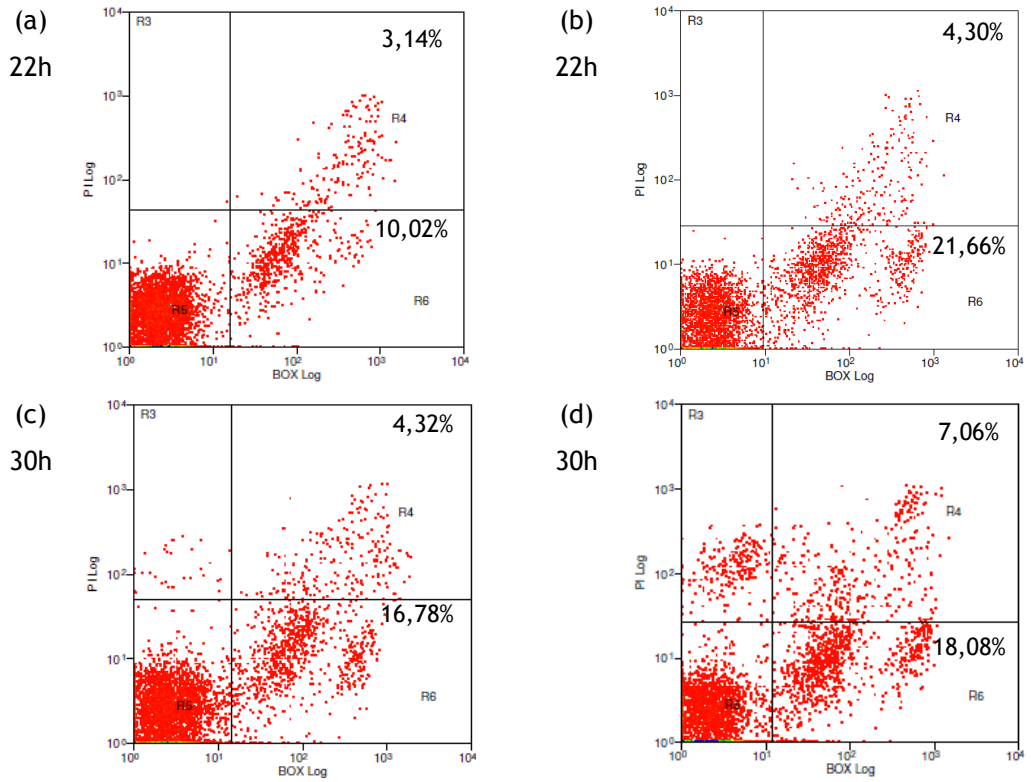


Figure 12. Fluorescence dot plots of cells (BOX: x-axis; PI: y-axis) taken during fermentation. The left column represents assay 4 (minimum of produced except assay 1) and the right column represents assay 8 (maximum of resveratrol produced).

In the figure 12 is possible to distinguish three populations. In quadrant R5 (lower left) are healthy cells, cells without PI or BOX. In R4 (upper right) are BOX/PI double positive cells, which have a depolarized and permeabilized membrane. In quadrant R6 (lower right) are depolarized cells labelled only with BOX, in other words, cells with its membrane depolarized. At first sample (with 22 hours of fermentation), the percentage of healthy cells was around 76 % and 8 hours later was 70 %. This can be explained mainly by the increase of depolarized cells (from 21 % to 26 %), once dead cells (PI positive) had a poor contribution to the final value (from 3.4 % to 4 %).

Although the vast majority of the cells were in a healthy state, this percentage is smaller when compared to the values obtained in other bioprocess monitoring studies [81]. The higher values of depolarized cells may be due to the fact that M9 medium is a minimal medium [91] (with only inorganic salts and a source of carbon), which limits nutrient availability, causing an increase in cell depolarization due to nutrient starvation [74], as cells at 22 and 30 h of fermentation were in the stationary phase. When the cells were more depolarized (> 30 %), it generally corresponded to lower resveratrol production, as seen in assays 18 and 19 (table 29). At 30 h of fermentation, 39.07 % of cells were depolarized in assay 19, where 15.90 µg/mL of resveratrol was obtained. The same relation is markedly noted in assay 18, where

14.90 µg/mL of resveratrol was achieved at 30 h when depolarization was 50.16 %. However, resveratrol production also depends on the growth conditions, since they could cause this molecule degradation in the culture medium. This can be seen in assay 22, which exhibited high depolarization but not a great decrease in resveratrol production, as 109.28 µg/mL of resveratrol were achieved although 31.11 % of cells were depolarized.

Generally, when the percentage of depolarized cells is above 30 %, it corresponds to assays where culture conditions did not favor resveratrol production. Temperature, as one of the most important factors in cell growth, also influenced cellular viability, as half of the assays with more than 30 % of depolarized cells were performed at 34 ºC (assays 14, 16, 18, 19, table 29) and 25 ºC (assay 24). Apparently, the precursor concentration seemed to affect cellular viability, as can be seen in assay 22, where the addition of 16 mM of *p*-coumaric acid provoked an increase in the percentage of depolarized cells. This decreased cellular viability can be due to the higher amount of DMSO added to the culture medium to attain a final precursor concentration of 16 mM, which may cause a destabilization of the cell membrane, leading to a reduction in the percentage of healthy cells [77]. Precursor addition at later stages of cell growth can also influence the physiological states, as seen in assay 23 (table 29), where *p*-coumaric acid was added at an OD_{600} of 1.025, when cells were in exponential phase, which led to an increase in the percentage of depolarized cells to 33.04 %. In the exponential phase, cellular growth is very fast [50] and the high concentration of DMSO added could damage the cellular membrane [77], which could break the transport balance [50]and lead to a lower concentration of resveratrol.

In conclusion, culture conditions affected cellular viability. High percentages of depolarized cells could not be sufficient to diminish resveratrol concentration, as seen in assay 22, where 31.11% of the cells at 30 hours of fermentation were depolarized but the amount of resveratrol produced was 109.28 µg/mL. This demonstrates that the culture conditions tested also lead to resveratrol degradation in culture medium, concealing the effect of cell viability on resveratrol production.

3.6. Plasmid segregational stability

The assessment of plasmid segregational stability provides new insights and allows a more comprehensive approach of the fermentation bioprocesses and the results obtained for PCN quantification are presented in table 30. In these tests, real-time qPCR was used to determine PCN at 22 and 30 hours of fermentation, an important factor to determine the viability of the cells and consequent productivity.

Table 30. Effect of growth conditions on plasmid copy number at 22 and 30 h of fermentation.

Run	pAC-4CL1		pUC-STS	
	22h	30h	22h	30h
1	117	53	31	62
2	30	47	23	14
3	29	77	22	35
4	84	44	40	21
5	51	62	15	14
6	204	85	65	16
7	218	394	91	64
8	215	1541	72	89
9	115	130	20	26
10	53	31	33	14
11	81	113	44	69
12	42	55	32	25
13	58	55	15	17
14	55	96	28	33
15	28	40	4	4
16	64	94	69	59
17	38	37	48	37
18	75	107	71	88
19	48	36	10	5
20	64	77	22	33
21	34	44	78	160
22	48	67	103	135
23	64	81	177	239
24	26	28	25	30
25	3	2	14	40
26	51	82	128	206
27	17	14	48	39
28	2	3	2	3
29	33	36	45	62
30	47	77	95	86

In a production bioprocess, plasmid amplification leads to increase plasmid DNA yields, which can result in higher plasmid segregational stability [87] and consequently increase product yield [78]. In this way, measuring plasmid segregational stability through PCN variation throughout the fermentation provides new insights and allows a more comprehensive approach of the fermentation bioprocesses, helping to define the best conditions to obtain the highest yield.

In the majority of these assays, PCN increases both in pAC-4CL1 and pUC-STS from 22 to 30 h (table 30), which could partially explain the higher resveratrol production yields also obtained in the samples taken after 30h of fermentation. The plasmids used have two different origins of replication: plasmid pAC-4CL1 has a p15A origin, while plasmid pUC-STS has a pBR322 origin of replication. Both these plasmids origins of replication are inducible at temperatures ranging from 37 to 42 ºC, but not 30 – 32 ºC [92, 93]. In this study, when resveratrol production is low, the temperature is almost always 34 ºC, which is not an inductive temperature for these origins of replication, leading to lower plasmid amplification, as seen in previous studies [87]. Absolute values of PCN from pUC-STS are also lower in comparison with pAC-4CL1 values. These are expected values for this temperature-sensitive plasmid pUC-STS, because the tested temperatures did not favored their induction, which together with the genes for ampicillin resistance [74] increased the segregational instability [81].

In general, these PCN values are low if compared with other studies [78, 81], and an explanation could be related with the metabolic burden imposed to the *E. coli* cell by the maintenance and replication of two plasmids which resulted in lower cell growth and PCN values, indicating a possible increase in plasmid segregational instability [74].

Chapter 4

4. Conclusions

Resveratrol, a secondary metabolite, is present in several plants and due to its importance, was used as herbal folk remedy for centuries. Several health benefits have currently been attributed to resveratrol, as cardioprotective benefits (French Paradox), neuroprotetive, cancer chemoprotective, antimicrobial among others. Being a compound of interest for the pharmaceutical and cosmetic industries, it is important to develop processes to keep up with the demand for this compound.

In this study, resveratrol was produced using two recombinant microorganisms (*Saccharomyces cerevisiae* and *Escherichia coli*) in bioreactors, after a set of screening assays, while cell physiology and plasmid segregational stability were monitored through flow cytometry and real-time qPCR, respectively, towards the improvement of bioprocess performance.

Resveratrol was quantified by an HPLC-DAD validated method, after an extraction with ethyl acetate. The method validation for resveratrol quantification was performed according to international guidelines and the results obtained were similar to other studies.

After a successful transformation of cells, several fermentations for screening out the best conditions for resveratrol production using *E. coli* and *S. cerevisiae* were carried out in shake-flasks. This was an innovator aspect of this work, because there is no literature with descriptions of extended assays to assess resveratrol culture conditions. In these studies, we can conclude that *E.coli* produce about 30 times more resveratrol than *S. cerevisiae*. It was also assessed that for *E.coli* the best precursor concentrations were 1, 5 and 10 mM. Concerning optical density (OD_{600}) at precursor addition, only densities above 0.2, had a final impact on resveratrol production. Resveratrol achieves its maximum production at pH 7 and optimal temperature is around 30 ºC for both microorganisms. In *S. cerevisiae*, the maximum production was achieved at 5 mM of precursor and it seems that neither the optical density at time of induction nor the changes in medium increase resveratrol production. It was also seen that the agitation speed and medium composition did not have a significant influence on the overall resveratrol yield for both microorganisms. At the end, *E. coli* produced as much resveratrol as already described for this strain (159.96 µg/mL) and *S. cerevisiae*, in some assays, produced more resveratrol than described in some studies (3.17 µg/mL).

In bioreactor, although the experimental design validation was not achieved, a maximum of 159.96 µg/mL resveratrol was obtained which corresponds to the highest resveratrol yield to date for this recombinant strain. The optimal conditions that allowed this resveratrol yield

were 28 ºC, pH 6.5 at OD_{600} 0.8 and 4 mM of *p*-coumaric acid. The media used was M9, an economically viable media. These assays highlighted that an factorial approach, through the addressing of several factors at a time is more useful than one-factor-at-a-time approach, because it can provide better results due to the assessment of the simultaneous influence of various factors on overall production.

The assessment of physiological state of the cells is important to monitor the bioprocess. Flow cytometry is a fast analysis technique and an adequate method to achieve a huge amount of multiparametric information. At the end of fermentation, the percentage of viable cells was about 70 %, due to the presence of a high percentage of depolarized cells because cellular viability is known to decrease toward the end of fermentation, due to the depletion of nutrients, leading to a lower cellular activity and metabolic energy (to maintain cellular functions) and consequently to cell depolarization. Nevertheless, one of the plasmids in the studied *E. coli* was high copy plasmid, which is related to a induced metabolic burden and probably promoted cellular depolarization. Temperature is slightly prejudicial to cells and the culture conditions tested also lead to resveratrol degradation in culture medium, concealing the effect of cell viability on resveratrol production.

Another simple technique to assess segregational instability and monitor the production process was the evaluation of plasmid copy number by real-time qPCR. In these assays, PCN increases both in pAC-4CL1 and pUC-STS from 22 to 30 hours. However, both plasmids are induced by temperature in values that were not tested in these assays, which led to plasmid segregational instability. Furthermore, pUC-STS, contains an ampicillin resistance gene and is more prone to segregational instability. However, the amounts of PCN obtained were lower than the ones obtained by other studies. Analyzing this data along the data obtained by flow cytometry, the viability of the cells is similar to other studies and due to stress, cellular viability is reduced and slightly influences plasmid segregational stability.

In sum, resveratrol production in bioreactor was carried out successfully. Adequate tools were used to monitor the overall process which produced a large quantity of this stilbene in an economically viable way. This study can be a contribution to a possible starting point of industrial resveratrol production and an effective alternative to chemical synthesis and also to avoid the depletion of the natural sources of this compound.

Chapter 5

5. Future perspectives

Resveratrol is one of the best know plant secondary metabolites. Due to its benefits to human health, it is one of the most promising plant-derived molecules. Despite the study performed, further research is needed in order to improve the whole bioprocess. As future prospects, the work may include the following topics:

- To develop a model for the bioprocess, including the interaction between culture conditions, cell physiology and plasmid stability;
- To create a new expression system with genes used to improve resveratrol production in *Escherichia coli* and *Saccharomyces cerevisiae*;
- To exploit various production strategies, for both microorganisms, as adding precursor several times in the same fermentation, fed-batch resveratrol production process or to test variations of physical factors, as temperature and pH, during fermentations;
- To develop sustainable downstream processes, in order to expand the existing industrial production.

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Appendix 1

Media Recipes

All the media presented here were sterilized (Uniclave 88, AJC, Portugal) and all the techniques were carried out in aseptic conditions.

LB

Per L of water: tryptone (Biokar Diagnostics, France), 10 g; yeast extract (Merck, USA), 5 g; NaCl (Panreac, Spain), 10 g. Adjust pH to 7.

SOC

Per L of water: tryptone, 20 g; yeast extract, 5 g; NaCl (VWR, USA), 10 g; KCl (Panreac, Spain) 250 mM, 10 mL. Adjust pH to 7. After autoclaving, add 5 mL of $MgCl_2$ 2M and 20 mL of glucose 20 mM (Sigma-Aldrich, USA).

SC

Per L of water: glucose (Sigma-Aldrich, USA), 20 g; yeast nitrogen base (Pronadisa, Switzerland), 6,7 g; tryptophan (Fluka, USA), 39,8 mg. Tryptophan should be dissolved in HCl (VWR, USA) 0,5M and added after autoclaving because it is thermosensitive.

M9

Per L of water: Na_2HPO_4 (Panreac, Spain), 6,779 g; KH_2PO_4 (Merck, USA), 3 g; NaCl, 0,5 g; NH_4Cl (Sigma-Aldrich, USA), 1 g; yeast extract, 1,25 g; glycerol (HiMedia, India), 5g. Adjust pH to 7. After autoclaving, add 2 mL of $Mg_2SO_4.7H_2O$ (Sigma-Aldrich, USA) 1M and 0,1 mL of $CaCl_2.2H_2O$ (Panreac, Spain) 1M.

YPD

Per L of water: yeast extract, 12 g; peptone (Biokar Diagnostics, France), 25g; glucose, 12 g. Adjust pH to 6,5.