Universidade do Minho
Escola de Engenharia

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Dissertação de Mestrado

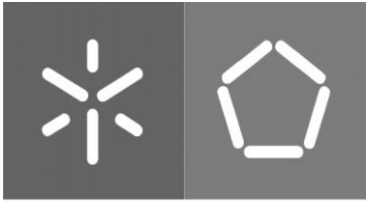
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação de

Professora Doutora Lígia Raquel Marona Rodrigues

Doutora Joana Lúcia de Lima Correia Rodrigues

Outubro de 2017



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Universidade do Minho, _____/_____/_____

Assinatura:

*"Man needs his difficulties because they are necessary to enjoy success"*

**A.P.J. Abdul Kalam**

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## ABSTRACT

Curcumin is a secondary metabolite produced by *Curcuma longa*. Studies have confirmed its biological and therapeutic effects in several diseases being the anticancer activity the most documented. Since curcumin is synthetized in low amounts, its heterologous production could represent a rapid and easy method to obtain large amounts of this bioactive compound. Curcumin has already been produced by *Escherichia coli*. However, the curcumin biosynthetic pathway has never been engineered in *Saccharomyces cerevisiae*. As a eukaryotic organism, it presents unique advantages over *E. coli* regarding the expression of plant derived genes. This work aimed to design, construct and validate a biosynthetic pathway composed by genes from different plants to produce curcumin from ferulic acid in an engineered *S. cerevisiae*. The enzymes involved in the artificial pathway are: 4-coumarate-CoA ligase (4CL) and different type III polyketide synthases (PKSs). In *C. longa* there are two types of PKSs - diketide-CoA synthase (DCS) and curcumin synthase (CURS) - that catalyse different reactions. Curcuminoid synthase (CUS) from *Oryza sativa* is also a PKS and catalysis the "one-pot" synthesis of curcuminoids in *E. coli*. Shuttle vectors with enzymes from different organisms were constructed and transformed into two *S. cerevisiae* strains. The vectors carried 4CL from *Arabidopsis thaliana* or *Lithospermum erythrorhizon*, and CUS or DCS and CURS. Curcumin was visually produced with a pathway composed by 4CL from *L. erythrorhizon* and CUS from *O. sativa*. The maximal curcumin production was 233.42 ng/L representing a yield of 1.46 %. Also, three different extraction methods were tested which revealed the importance of cell lysis in curcumin extraction from yeast. In addition, CRISPR-Cas9 method was used to knockout a gene from *S. cerevisiae* that codifies ferulic acid decarboxylase. As expected, the mutant strain was not able to consume ferulic acid. However, no curcumin production could be detected in the preliminary assays. It is important to mention that this study allowed to produce curcumin for the first time in an engineering yeast. In the future, optimizations at genetic and operational level are needed to improve the curcumin titters.



KEYWORDS: CURCUMIN, *SACCHAROMYCES CEREVISIAE*, CLONING, CRISPR-CAS9

# RESUMO

A curcumina é um metabolito secundário produzido pela *Curcuma longa*. Vários estudos confirmaram o seu efeito biológico e terapêutico em várias doenças sendo a atividade anticancerígena a mais documentada. Devido ao facto da curcumina ser sintetizada em pequenas quantidades, a sua produção heteróloga pode representar um método rápido e fácil para obter grandes quantidades deste composto bioativo. A curcumina já foi produzida em *Escherichia coli*. No entanto, a sua via biossintética nunca foi introduzida em *Saccharomyces cerevisiae*. Como organismo eucariótico, este apresenta vantagens únicas em relação a *E. coli* para expressão de genes derivados de plantas. Este trabalho teve como objetivo projetar, construir e validar uma via biossintética composta por genes de diferentes plantas para produzir curcumina a partir de ácido ferúlico em *S. cerevisiae*. As enzimas envolvidas na via artificial são: 4-cumárico-CoA ligase (4CL) e as policetídeo sintase tipo III (PKS). Em *C. longa* existem dois tipos de PKSs - diketide-CoA sintase (DCS) e curcumina sintase (CURS) - que catalisam reações diferentes. A curcuminoide sintase (CUS) da *Oryza sativa* é também uma PKS. Esta enzima foi capaz a sintetizar curcuminoides em *E. coli* num só passo. Para esse efeito, vetores "shuttle" com enzimas de diferentes organismos foram construídos e transformados em duas estirpes de *S. cerevisiae*. Os vetores possuíam 4CL de *Arabidopsis thaliana* ou de *Lithospermum erythrorhizon*, e CUS ou DCS e CURS. Foi possível aferir visualmente a produção de curcumina nas leveduras onde foi inserida uma via composta por 4CL de *L. erythrorhizon* e CUS de *O. sativa*. A produção máxima de curcumina foi de 233,42 ng/L, o que representa um rendimento mássico de 1,5%. Além disso, três métodos de extração diferentes foram testados, o que demostrou a importância da lise celular para extração de curcumina em levedura. Além disso, o método CRISPR-Cas9 foi usado para silenciar um gene em *S. cerevisiae* que codifica a ácido ferúlico descarboxilase. Como esperado, o mutante resultante não foi capaz de consumir ácido ferúlico. No entanto, em ensaios preliminares também não foi possível detetar a produção de curcumina. É de realçar que este foi o primeiro trabalho em que a curcumina foi produzida por uma levedura geneticamente modificada. No futuro, são necessárias otimizações a nível genético e operacional por forma a aumentar os rendimentos de produção.

PALAVRAS CHAVE: CURCUMINA, *SACCHAROMYCES CEREVISIAE*, CLONAGEM, CRISPR-CAS9

# SCIENTIFIC OUTPUT

**International Conference Oral Communication:**

Rainha J., Couto MR., Rodrigues JL., Rodrigues LR. Production of curcumin from ferulic acid by an engineered *Saccharomyces cerevisiae.* II International Congress in Health Sciences Research. 2017. May 17. University of Beira Interior, Covilhã, Portugal.

**National Conference Communication:**

Rodrigues JL., Couto MR., Rainha J., Rodrigues LR. Synthetic biology: Heterologous production of bioactive agents. Centre of Biological Engineering Annual Meeting. 2017. July 6. University of Minho, Braga, Portugal.

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# ABBREVIATIONS

| | |
|---|---|
| 4CL | 4-coumarate-CoA ligase |
| ACC | Acetyl-CoA carboxylase |
| AP-1 | Activator protein 1 |
| Asn | Asparagine |
| C4H | Cinnamate-4-hydroxylase |
| CCoAOMT | Caffeoyl-CoA O-methyltransferase |
| CDK | Cycling dependent kinase |
| CHO | Chinese hamster ovarian cell |
| CoA | Coenzyme A |
| COMT | Caffeic acid O-methyltransferase |
| COX2 | Cycle oxigenase 2 |
| CQT | *p*-Coumaroyl quinate |
| CST | *p*-Coumaroyl shikimate transferase |
| CURS | Curcumin synthases |
| CUS | Curcuminoid synthase |
| Cys | Cysteine |
| DCS | Diketide-CoA synthase |
| FDC | Ferulic acid decarboxylase |
| FGF | Fibroblast growth factor |
| GRAS | Generally regarded as safe |
| His | Histidine |
| HIV | Human Immunodeficiency Virus |
| HPLC | High-performance liquid chromatography |
| HPTLC | High-performance thin-layer chromatography |
| iNOS | Inducible nitric oxide synthase |
| JEFCA | Joint FAO/WHO Expert Committee on Food Additives |
| JNK | Jun N-terminal kinases |
| $K_{cat}$ | Turnover rate |
| $K_m$ | Michaelis-Menten constant |
| LB | Lysogeny broth |

| | |
|---|---|
| LTR | Long terminal repeat |
| MMP | Matrix metalloproteinase |
| MRP | Multidrug resistance related proteins |
| MS | Mass spectrometry |
| NAD(P)H | Nicotinamide adenine dinucleotide phosphate |
| NF-κb | Nuclear factor κB |
| $OD_{600nm}$ | Optical density at 600 nm |
| PAD | Phenylacrylic acid decarboxylase |
| PAIN | Pan-Assay Interference Compound |
| PAL | Phenylalanine ammonia lyase |
| PCR | Polymerase chain reaction |
| PKS | Polyketide synthase |
| PLGA | Poly (lactic-co-glycolic acid) |
| PPAR-γ | Peroxisome proliferator activated receptor γ |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| STS | Stilbene synthase |
| TAL | Tyrosine ammonia lyase |
| TBARS | Thiobarbituric acid reactive substances |
| TFA | Trifluoroacetic acid |
| TNF-α | Tumor necrosis factor α |
| UV | Ultra-violet |
| VEGF | Vascular endothelial growth factor |
| yEP | Yeast episomal plasmids |
| yIP | Yeast integrative plasmids |
| YNB | Yeast nitrogen base |
| YNB -URA | Yeast nitrogen base with the required nutrients lacking uracil |
| YNB +URA | Yeast nitrogen base with the required nutrients including uracil |
| YPD | Yeast extract peptone dextrose |

# SCOPE

Cancer has a major impact on society being responsible for 8.8 million of deaths in 2015 and representing the second leading cause of death worldwide. The number of new cases is expected to rise by about 70 % over the next two decades (World Health Organization, 2017). Great efforts have been made to find therapeutic compounds and ways to produce them to reduce cancer mortality.

Curcumin has been studied extensively due to its anticancer activity. Its effect during the various stages of carcinogenesis have been reported, pointing this compound as a potential natural drug for cancer treatment. Apart from its anticancer activity, curcumin seems to be a very interesting molecule because it presents other biological activities. However, curcumin extraction from plants is time-consuming and inefficient since pure curcumin is not obtained. Moreover, it is hard to chemically synthesize hampering its utilization. The use of microorganisms to produce curcumin presents itself as an innovative and economical solution. The yeast *Saccharomyces cerevisiae,* besides the extensive toolbox of genetic modifications and fermentative strategies, possesses the GRAS classification which facilitates further applications in the pharmaceutical industries. The current work was developed under this scope, aiming the production of curcumin by an engineered yeast.

# 1 STATE-OF-THE-ART

## 1.1 Curcumin

The secondary metabolism refers to the metabolic pathways that lead to compounds that are not involved in the processes of growth, development and reproduction (Kossel, 1891). Plants produce a wide variety of secondary metabolites and such compounds are characterized by their low abundance and also to be stored in specific organs or cells. They are responsible for plant adaptation to the environment acting as protectors against pathogens and animals, absorbing UV light, among others (Bourgaud et al., 2001). These compounds are classified according to their biosynthetic pathway and are divided in three large families: phenolics, terpenes and steroids, and alkaloids (Harborne and Baxter, 1999). For centuries, the secondary metabolites have been used in traditional medicine due to their biological activity, and nowadays they are also used in pharmaceutics, cosmetics, fine chemistry and others (Bourgaud et al., 2001).

Turmeric (*Curcuma longa*) is a perennial herb native from India belonging to ginger family and extensively cultivated in southeast tropical Asia. Its rhizome is the most useful part and has been used in culinary as spice, in traditional medicine and as coloring agent in foods and textiles. Curcuminoids are secondary metabolites produced by *C. longa* and represent the most active compounds of the plant. They have been widely used as yellow food coloring, in drugs and cosmetics (Aggarwal et al., 2007). Chemically, they are classified as polyphenols more specifically diarylheptanoids. There are three major types of curcuminoids in *C. longa*: curcumin, demethoxycurcumin and bis-demethoxycurcumin (**Figure 1**). In general, the term curcumin is used to designate all the curcuminoids (Anand et al., 2008). Curcumin [IUPAC name (1E, 6E)-1,7-bis-(4-hydroxy-3-methoxy-phenyl)-1,6-heptadien-3,5-dione; CAS number: 458-37-7] is an orange-yellow crystalline powder water insoluble (but soluble in ethanol and other organic solvents) and was described in 1910 by Lampe and Milobedeska. The molecular formula of curcumin is $C_{21}H_{20}O_6$ and it has a molecular weight of 368.37g/mol. Today there are more than 120 known species of *Curcuma* and pure curcumin is obtained from them by solvent extraction and subsequent crystallization. The amount of curcuminoids that can be

extracted varies among different species and it depends heavily on the geographical conditions (Esatbeyoglu et al., 2012).

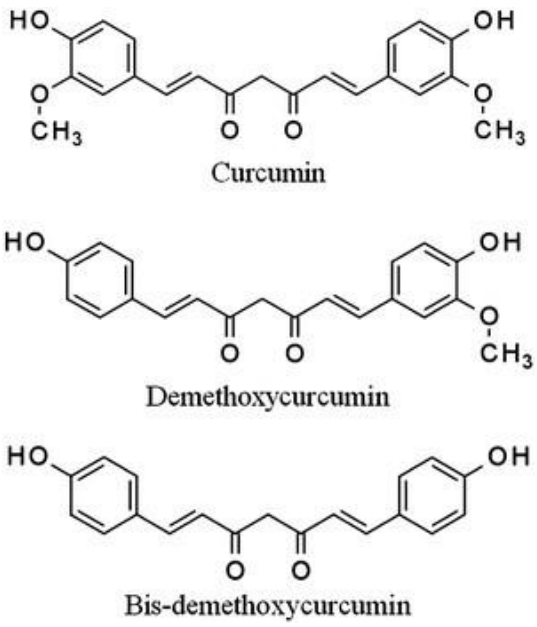



**Figure 1:** Structures of curcumin, demethoxycurcumin and bis-demethoxycurcumin.

### 1.1.1 Biological activity

Curcumin was first isolated in 1815 by Vogel and Pelletier. Later in 1937 the first study of its effect in human disease was published, and in 1949 its antibacterial activity was reported by Schraufstätter and Bernt. In the last five decades, several studies confirmed the biological effects of curcuminoids in humans and its therapeutic effects were widely described making curcumin one of the most studied naturally-derived therapeutic products (Aggarwal et al., 2007; Epstein et al., 2010; Esatbeyoglu et al., 2012; Joe et al., 2004; Maheshwari et al., 2006; Zhang et al., 2013).

#### 1.1.1.1 Anticancer activity

The effect of curcumin has been studied in various human carcinomas, namely liver (Chuang et al., 2000), skin (Huang et al., 1995; Jiang et al., 2015), pancreatic(Li et al., 2005), prostate (Dorai et al., 2001), ovarian (Cai et al., 2013), lung (Yang et al., 2012), head and neck (Azuine and Bhide, 1994; Tanaka et al., 1995) cancers, among others. The anticancer activity of this compound is diverse (**Table 1**), targeting various levels of regulation during the various stages of carcinogenesis from DNA mutation to the metastasis and apoptosis (Perrone et al., 2015; Wilken et al., 2011).

#### 1.1.1.2 Other biological activities

Several studies confirmed other curcumin biological activities besides anticancer. The antioxidant activity is one of the most documented effects of curcumin and its derivatives. Curcumin showed to act as antioxidant inhibiting lipid peroxidation and other oxidative damages. For example, Unnikrishnan and Rao (1995) concluded that curcumin provided a protection to hemoglobin from oxidation induced by nitrogen dioxide. The antioxidant effect is related to the presence of phenolic and methoxy groups that contribute to the free-radical-scavenging (Ruby et al., 1995; Sharma, 1976; Sugiyama et al., 1996). Curcumin showed the strongest free-radical-scavenging activity relatively to other curcuminoids due to its electrochemical properties (Motterlini et al., 2000). In addition to antioxidant activity, curcuminoids present numerous properties that are beneficial to human health (**Table 2**).

**Table 1:** Some curcumin effect in anticancer activity.

| | **Curcumin effect description** | **References** |
|---|---|---|
| **Effect on transcription factors** | -Suppresses the expression of gene products involved in carcinogenesis and tumor growth (such as COX2); | (Weir et al. 2007; Park et al., 2005; Tharakan et al. 2010) |
| **Effect on cell cycle regulation** | -Upregulates the expression of the Cip/Kip family of CDK inhibitors, thus inhibiting the association of cyclin D1 with CDK4 and CDK6;<br>-Decreases phosphorylation of Rb and suppresses transcription of E2F-regulated genes; | (Park et al. 2002); Mukhopadhyay et al. 2002) |
| **Effect on apoptosis** | -Selectively induces apoptosis in G2 phase tumor cells via upregulation of p53 expression and initiation of mitochondrial apoptotic pathway via increased Bax expression and cytochrome c release;<br>-Stimulatory effect in extrinsic apoptosis, triggered by the binding of "death activators" such as TNF-α and Fas Ligand to their cell surface receptors;<br>-Promotes the aggregation of Fas receptors and increases the levels of caspase-8 and -3; | (Bush et al., 2001; Wang et al., 1995; Weir et al., 2007) |
| **Effect in autophagic cell death** | -Inhibits the Akt/mTOR/p70S6 kinase pathway and ERK1/2 pathway, involved in the regulation of autophagy induced by nutrient stress leading to G2/M cell cycle arrest and non-apoptotic autophagic death; | (Jia et al., 2009; O'Sullivan-Coyne et al.,, 2009) |
| **Effect on angiogenesis and metastasis** | -Regulates a variety of pro-angiogenic growth factors, enzymes and transcription factors including bFGF, VEGF, angiopoetin-1 and 2, COX-2, MMP-9, AP-1 and NF-κB;<br>-Inhibits the angiogenic response to FGF-2 stimulation;<br>-Decreases the levels of the angiogenic biomarkers COX-2 and VEGF;<br>-Affects cellular adhesion molecules involved in the processes of tumor growth and metastasis | (Gururaj et al., 2002; Mohan et al., 2000; Ray et al., 2003; Yoysungnoen et al., 2006) |

**Table 2**: Some of the therapeutic effects of curcumin.

| | **Curcumin effect description** | **References** |
|---|---|---|
| **Anti- inflammatory** | -Inhibits molecules that mediate the inflammatory response like COX-2, iNOS through suppression of NF-κB; | (Jobin et al., 1999) |
| **Anti-diabetic** | -Inhibits nuclear factor kappa beta;<br>-Inhibits protein carbonyl, lipid peroxidation and lysosomal enzyme activities;<br>-Decreases the levels of TBARS (Thiobarbituric acid reactive substances) and the activity of SDH (Sorbitol dehydrogenase);<br>-Induces PPAR-γ (peroxisome proliferator activated receptor gamma);<br>-Can elevate plasma insulin level and increase lipoprotein lipase activity;<br>-Activates enzymes associated with glycolysis, glycogenic and lipid metabolism in liver; | (El-Moselhy et al, 2011; He et al., 2012; Seo et al., 2008) |
| **Anti-Alzheimer's** | - Decreases the accumulation of amyloid β-peptide, and markers of oxidative stress and inflammation in the cerebral cortex;<br>- Stimulates neuroplasticity by acting on Nrf2 and promoting the expression of superoxide dismutase, catalase, sulfaredoxin, thioredoxin, peroxiredoxin systems, glutathione peroxidase, glutathione reductase, γ-glutamine cysteine ligase and γ-glutamine cysteine synthase, quinone recycling (NAD(P)H quinone oxidoreducase, and heme oxygenase 1; | (Farooqui, 2016; Yang et al., 2005) |
| **Anti-Parkinson** | - Inhibits aggregation of α-synuclein;<br>- Regulates proteins involved in iron metabolism;<br>- Inhibits JNK (c-Jun N-terminal kinases) phosphorylation; | (Mythri and Bharath, 2012; Wang et al., 2010) |
| **Anti-HIV** | -Inhibits HIV-1 LTR gene expression;<br>-Inhibits p24 antigen production through transcriptional repression of LTR;<br>-Inhibits HIV-1 and HIV-2 proteases activities;<br>-Inhibits HIV-1 integrases activity;<br>-Inhibits the acetylation of HIV-Tat protein. | (Abraham et al., 1993; Mazumder et al., 1997) |

In addition to the properties reported in **Table 2** there are many more documented biological and medical properties of curcumin in arthritis, pancreatitis, muscle regeneration, cystic fibrosis, against cataract formation, in lung and liver injuries, wound healing and scarring and in cholesterol lowering and cardiovascular diseases among others (Aggarwal et al., 2007).

However, contradictory information regarding curcumin biological activities had been discussed, mainly in the review article by Nelson et al. (2017). The authors questioned first the use of different types of curcumin in *in vivo* tests and clinical trials, usually a curcuminoid mixture, while in *in vitro* tests, generally pure synthetic curcumin is used. Secondly, they mentioned that curcumin appears to be a PAIN (pan-assay interference compound) exhibiting all known PAIN-type behaviour like covalent labelling of proteins, membrane disruption, fluorescent interference and structural decomposition causing false-positive results in *in vitro* assays. Despite the reported biological activities in the literature, the authors referred its low half life time at physiological conditions, its photoreactivity, solvolysis and oxidative degradation responsible for the misleading results because the biological effect may be caused by a degradation product. Curcumin also displays undesirable physiochemical properties relative to known drugs like forming chemical aggregates and water insolubility. Regarding pharmacokinetic and pharmacodynamic properties the authors concluded that they are poor due to low absorption, distribution in the body, high potential to be metabolized and excreted. Also, curcumin exhibited toxicological effects due its iron chelation activity. The authors also referred that curcumin never shown to be conclusively effective in a randomized, placebo-controlled clinical trial and concluded that curcumin is “reactive, nonbioavailable and therefore a high improbable lead” compound. However, Padmanaban and Nagaraj (2017) state that the conclusions taken by Nelson et al. (2017) cannot be drawn and show several examples, including clinical trials, where significant beneficial effect of curcumin was observed. In conclusion, the authors agree that a cautionary approach is welcome, but that curcumin has an enormous potential.

### 1.1.2 Bioavailability

Curcumin is considered as a safe and novel drug for the treatment of a wide range of diseases. In numerous phase I studies, adverse effects were not observed in humans taking up to 12 g of curcumin per day orally (Vareed et al., 2008). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) set an acceptable daily intake value of 0-3 mg/kg body weight per day for curcumin in 2004. In 2009, Sabinsa Corporation received a "generally regarded as safe" (GRAS) status for the branded ingredient Curcumin C3 Complex® (Majeed et al., 2002) after a review of safety and toxicology data by Soni & Associates, Inc.

Studies on bioavailability of curcuminoids have been performed in numerous investigations in rodents and also in humans. Despite curcumin presenting a wide range of therapeutic applications, its bioavailability is low due to a relatively low absorption in intestine, rapid metabolism in liver and elimination through the gall bladder if administered orally. It is only significantly detected in plasma or in tissues after administration of high oral doses (Yang et al. 2007). Curcumin is conjugated with glucuronic acid and sulfate in enterocytes, these conjugates are actively transported out of the cells through the multidrug resistance related proteins (MRP) which accounts for its low bioavailability (Wortelboer et al., 2003).

To solve the curcumin low bioavailability, it is necessary to improve its solubility in aqueous solutions. The solubility of curcumin can be increased by the formation of complexes with metal ions. More recently, other methods showed to improve curcumin bioavailability using piperine, nanoparticles, liposomes or phospholipids (Basnet and Basnet, 2011). Piperine works as an adjuvant that blocks the curcuminoids metabolism. However, the use of adjuvants may represent a problem because a lot of xenobiotics are detoxified by the same pathway (Shoba et al., 1998). Therefore, new approaches are being tested to delivery drugs using targeted drug delivery system such as nanoencapsulation. Xie et al. (2011) confirmed that the use of PLGA (poly (lactic-co-glycolic acid)) nanoparticles elevates the oral bioavailability of curcumin. The same results were obtained using lecithin liposomes and cyclodextrin. In addition, the use of analogs of curcumin demonstrated to increase the bioavailability and enhanced the therapeutic effects (Yallapu, et al. 2010; Takahashi, et al. 2009).

## 1.2 Curcuminoid biosynthetic pathway in *Curcuma longa*

Ramirez-Ahumada et al. (2006) reported the first work that identified the enzymes involved in the synthesis of curcumin confirming the involvement of the phenylpropanoid pathway that channels the carbon flow from primary metabolism to different branch pathways of secondary metabolism which includes the synthesis of curcumin. Curcumin, as also other curcuminoids, consists of two phenylpropanoid units derived from phenylalanine connected by a central carbon derived from malonyl-CoA and this reaction is catalysed by type III PKS (Polyketide synthase) enzymes.

The enzyme phenylalanine ammonia lyase (PAL) is the first enzyme of this pathway and it represents a branch point between primary (amino acids metabolism) and secondary metabolism (phenylpropanoid pathway). It catalyses the formation of cinnamic acid that is converted to *p*-coumaric acid by cinnamate-4-hydroxylase (C4H). Next, there are two different ways for *p*-coumaric acid metabolism. First, it may be converted in other hydroxycinnamic analogues namely ferulic acid by 4-coumarate 3-hydroxylase (C3H) and caffeic acid O-methyltransferase (COMT) having caffeic acid as intermediate, or it can be activated by condensation with malonyl-CoA to form the corresponding CoA ester. This reaction is catalysed by 4-coumarate-CoA ligase (4CL). 4CL acts in multiple substrates, activating also the other analogues. After activation, *p*-coumaroyl-CoA can also be converted to the other activated analogues, caffeoyl-CoA and feruloyl-CoA by a set of enzymes which include *p*-coumaroyl shikimate transferase (CST) and caffeoyl-CoA O-methyltransferase (CCoAOMT). The CoA esters are converted to curcuminoids by PKS. However, Ramirez-Ahumada et al., (2006) despite detectable curcuminoid synthase activity in *in vitro* assays, were not able to characterize the enzyme(s) responsible for the reaction. The CoA esters are essential intermediates not only in the biosynthesis of curcumin but also in the biosynthesis of other plant phenolic secondary metabolites exalting the importance of the phenylpropanoid pathway in plant secondary metabolism (Ehlting et al., 1999).

Furthermore, Katsuyama and collaborators (2007) proposed a pathway for the curcuminoids synthesis in *C. longa* including two type III PKS. One, named diketide-CoA synthase (DCS), catalyses the formation of feruloyl-diketide-CoA from feruloyl-CoA and malonyl-CoA. The other, curcumin synthase 1 (CURS1), catalyses the hydrolysis of feruloyl-diketide-CoA in a β-keto acid and condensates it with other molecule of feruloyl-CoA forming

curcumin. DCS and CURS1 also accept *p*-coumaroyl-CoA to form bis-desmethoxycurcumin but with low efficiency. Later, the same authors (Katsuyama et al. 2009) characterized two other type III PKS, CURS2 and CURS3. *In vitro* analyses confirmed distinct substrates specificities for these enzymes. CURS2 uses preferentially feruloyl-CoA while CURS3 uses feruloyl-CoA and also *p*-coumaroyl-CoA. The existence of three different CURS could be the reason to explain the distribution of three curcuminoids in *C. longa*. CURSs and DCS share a conserved Cys-His-Asn catalytic triad and more than 60% identity (**Table 3**).

**Table 3**: Identity and kinetic parameters of DCS, CURS1, CURS2 and CURS3 from *Curcuma longa.*

| Enzyme | Identity w/ DCS | Identity w/ CURS1 | Identity w/ CURS2 | Identity w/ CURS3 | Subtract | $K_m$ (µM) | $K_{cat}$ ($s^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|---|
| **DCS** | | 62% | 62% | 66% | Feruloyl-CoA | - | 0.020 | (Katsuyama et al. 2009a; Katsuyama et al. 2009b) |
| | | | | | Malonyl-CoA | 8.4 | 0.011 | |
| **CURS1** | 62% | | 78% | 81% | Feruloyl-CoA | 18 | 0.018 | |
| | | | | | Coumaroyl-CoA | 189 | 0.014 | |
| **CURS2** | 62% | 78% | | 81% | Feruloyl-CoA | 4.3 | 0.007 | |
| | | | | | Coumaroyl-CoA | 89 | 0.016 | |
| **CURS3** | 66% | 81% | 81% | | Feruloyl-CoA | 2.2 | 0.003 | |
| | | | | | Coumaroyl-CoA | 2.4 | 0.006 | |

Curcuminoid biosynthetic pathway in *C. longa* is represented in **Figure 2**.

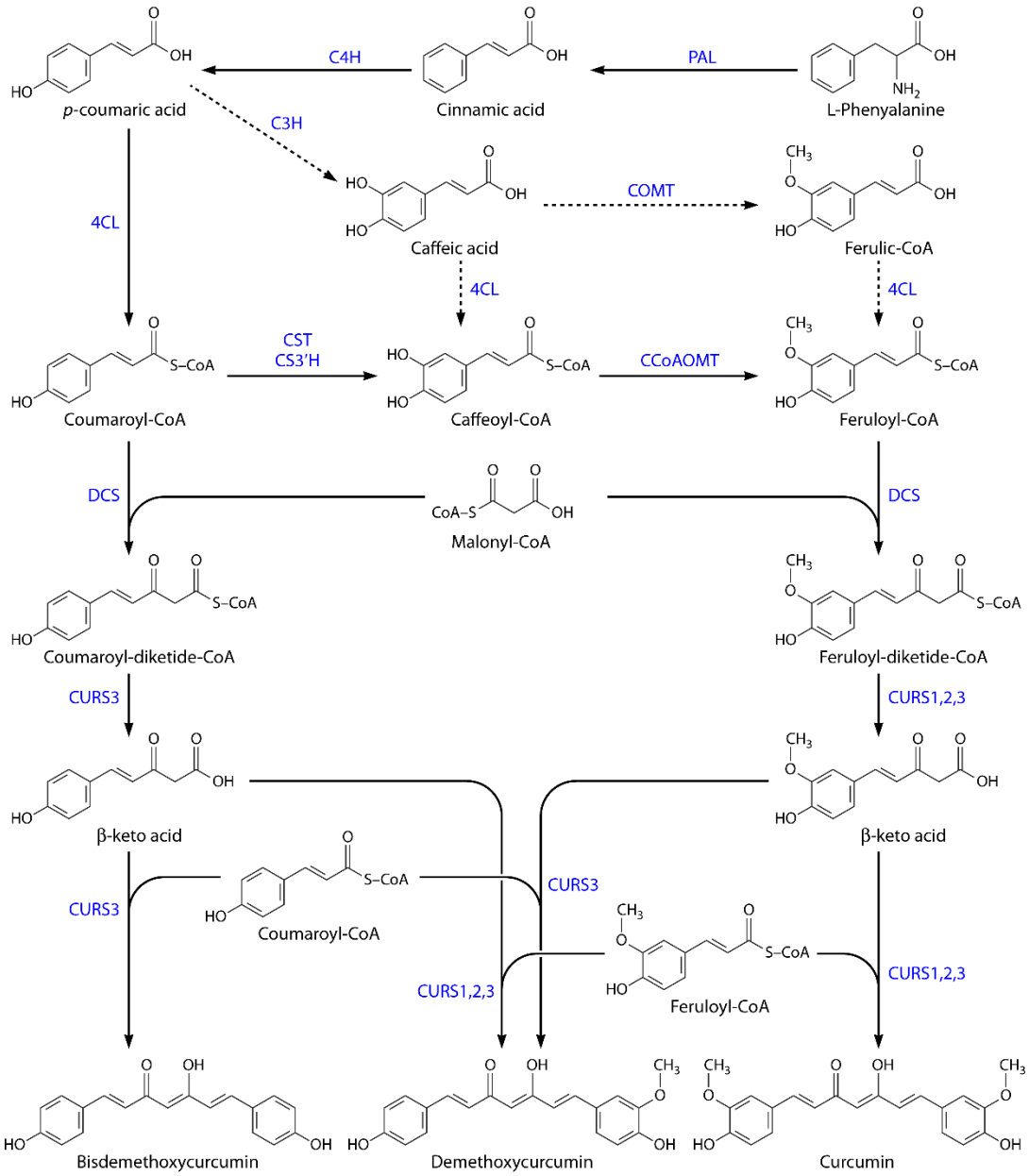



**Figure 2**: Curcuminoid biosynthetic pathway in *Curcuma longa*. Cinnamic acid is synthesized from phenylalanine by phenylalanine ammonia lyase (PAL) and converted to p-coumaric acid by cinnamate-4- hydroxylase (C4H). Then, 4-coumarate-CoA ligase (4CL) converts p-coumaric acid to p-coumaroyl-CoA and p-coumaroyl shikimate transferase (CST), p-coumaroyl 5-O-shikimate 3'-hydroxylase (CS3'H) and caffeoyl-CoA O-methyltransferase (CCoAOMT) converts it to feruloyl-CoA. p-coumaroyl-CoA and feruloyl-CoA are then converted by diketide-CoA synthase (DCS) to diketide-CoAs by condensation with malonyl-CoA. In the end, curcumin synthases (CURSs) catalyze the formation of curcuminoids by condensing the diketide-CoAs with p-coumaroyl-CoA and feruloyl-CoA. Depending on the combination, different curcuminoids are produced, namely bis-demethoxycurcumin, demethoxycurcumin and curcumin. The route indicated by dashed arrows corresponds to a less central phenylpropanoid pathway and may not occur in vivo in C. longa (taken from Rodrigues et al. 2015).

## 1.3 Heterologous production of curcuminoids

Plant secondary metabolites, such as curcuminoids, are accumulated in low quantities, are very difficult to isolate and hard to chemically synthesize. Given all the benefits of curcuminoids it is necessary to produce these compounds in significant amounts and preferentially in an inexpensive way. The heterologous production seems to be the better approach for this purpose once. In addiction, heterologous production has the advantage of producing pure curcumin as opposed to when it is extracted from turmeric where a curcuminoid mixture is obtained.

Heterologous production in plants, on the first hand, has the advantage of requiring the introduction of only one or two genes because the other genes from the phenylpropanoid pathway are already present. Moreover, unlike bacteria, they are capable of eukaryotic post-translational modifications, such as glycosylation. On the other hand, transgenic plants are not well accepted in the public domain and genetically modified crops have high costs (Streatfield, 2007). However, cultures of plant cells have been used to investigate the production of secondary metabolites and plant-cell models carried-out in liquid medium are being developed like hairy roots or cell suspension cultures. These models proved to be adaptable to large scale reactor. For example, various plants have been used for the production of *trans*-resveratrol constitutively in *in vitro* conditions (Streatfield, 2007). The concentrations of *trans*-resveratrol that can be produced with the use of plant cell cultures is at least equal to that reported as naturally occurring in the plant (Donnez et al., 2009).

The use of microorganisms has several advantages because they can grow in inexpensive substrates, are easier to manipulate and have fast production cycles allowing a faster and larger production. Also, microbes usually do not have competing pathways to transgenic metabolism making large-scale fermentation and downstream purification easier (Jeandet et al., 2012). In addition, the regulations for genetically modified microorganisms are simpler than those for plants (Key et al. 2008).

Mammalian cells represent another alternative host for the curcuminoid synthesis. The gene expression in mammalian cells needs a suitable cell line being the most widely used the Chinese hamster ovary (CHO). The use of animal cells has the advantage of enabling post-translational modifications, an accurate folding, an efficient assembly of subunits and the secretion of recombinant proteins. Nevertheless, the costs associated with the use of

mammalian cells is high and not suitable to scale-up (Khan, 2013). Zhang et al. (2006) used unnatural fusing proteins and transformed mammalian cells (human HEK293 kidney cells) to produce 0.34 µg/mL (cell volume) of resveratrol. Besides that, no other experiments were conducted using animal cells to produce other polyketides.

Curcuminoids have already been produced in heterologous hosts. Katsuyama et al. (2008) constructed the biosynthetic pathway of curcuminoids in *E. coli* constituting the first study demonstrating the production of curcuminoids in a heterologous organism. PAL from the yeast *Rhodotorula rubra* was the first enzyme of this biosynthetic pathway. The carboxylic acids were converted to CoA esters by 4CL from *Lithospermum erythorhizon* and then in curcuminoids by an enzyme called curcuminoid synthase (CUS) from *Oryza sativa*. CUS is a type III PKS able of catalyzing the "one-pot" synthesis of bis-demethoxycurcumin from two molecules of *p*-coumaroyl-CoA and one molecule of malonyl-CoA. It catalyzes both steps catalyzed by DCS and CURS in *C. longa*. The Cys-His-Asn catalytic triad is conserved in CUS and this enzyme can also accept cinnamoyl-CoA and feruloyl-CoA as substrates to produce dicinnamoylmethane and curcumin, respectively. The architecture of CUS has already been studied by Morita et al. (2010), the authors proposed that CUS has a unique down-ward-expanding active-site architecture allowing the condensation reaction without cyclization. Therefore, the use of CUS is a simpler system than the DCS/CURS system. Besides that, PAL from *R. rubra* was shown to possess tyrosine ammonia lyase (TAL) activity, thus tyrosine can also be used as precursor. Katsuyama et al. (2008) also overexpressed acetyl-CoA carboxylase (ACC) from *Corynebacterium glutamicum* to increase the intracellular pool of malonyl-CoA. In another experiment, phenylpropanoid acids were supplied to a system with only 4CL, CUS and ACC genes removing the PAL step to increase the CoA ester concentration. Recombinant *E. coli* produced 91 ± 23 mg/L of bis-demethoxycurcumin, 84 ± 15 mg/L of dicinnamoylmethane, and 113 ± 22 mg/L of curcumin.

Rodrigues et al. (2015) also produced curcuminoids in recombinant *E. coli.* The researchers tested three different 4CL enzymes from two different organisms and different PKS for curcuminoids production (CUS, DCS and CURS). Ferulic acid and/or *p*-coumaric acid were used as precursors. The best results were obtained using 4CL1 from *Arabidopsis thaliana* and DCS and CURS1 from *C. longa* producing 70 mg/L of curcumin. This system was also tested to produce curcuminoids in a bioreactor (Rodrigues, 2014). The amount of produced curcumin was much higher than the amount produced of bis-demethoxycurcumin, suggesting that

feruloyl-CoA is the preferred substrate which is not in agreement with Katsuyama et al. (2008) results. In this work, the 4CL reaction was identified as the limiting step and the bioavailability of malonyl-CoA may have affected the amount of curcuminoids produced. More recently, Couto et al. (2017) using the same pathway and optimizing the fermentative conditions such as culture media or the inducer concentration, produced 353.4 mg/L which represents an yield of 95.93 %. Rodrigues et al. (2015) also produced curcumin using tyrosine through the production of caffeic acid as an intermediate.

Zhang et al. (2016) identified a new CURS enzyme from *Zingiber officinale*. This enzyme efficiently accepts 3-(4-hidroxyphenyl) propionyl-CoA to produce tetrahydro bisdemethoxycurcumin and also accepts the CoA esters used by CURS from *C. longa* to produce other curcuminoids. Moreover, this enzyme shares 81% identity with CURS1 and the highly conserved Cys-His-Asn catalytic triade is also present. In addition, the researchers constructed an unnatural fusion protein DCS::CURS and the transformed *E. coli* cells to produce curcumin from feruloyl-CoA.

More recently, Kim et al. (2017) designed a biosynthetic pathway for *de novo* synthesis of curcuminoids in *E. coli*. Two pathways were constructed; the first containing 4CL and CUS from *O. sativa* controlled by different promoters and another with the same genes regulated by the same promoter. The TAL gene from *Saccharothrix espanaensis* was also inserted together with 4CL and CUS. The engineered *E. coli* strains produced 0.32 mg/L and 0.09 mg/L of bisdemethoxycurcumin, respectively. After that, the curcuminoid productivity was also tested using a genetic modified *E. coli* strain for tyrosine overproduction. The same enzymes combination was used and the mutants produced a maximum yield of 6.02 mg/L. Besides that, dicinnamoylmethane, other curcuminoid, was produced from phenylalanine cloning PAL from *A. thaliana* in combination with 4CL and CUS from *O. sativa*.

## 1.4 Engineering *Saccharomyces cerevisiae*

The curcuminoids biosynthetic pathway has never been engineered in *S. cerevisiae* or in another organism apart from *E. coli.* Hereupon, engineering a new yeast to produce curcuminoids is an attractive approach. *S. cerevisiae* was the first eukaryotic organism whose genome was fully sequenced (Goffeau et al., 1996) and has been engineered to produce a

wide variety of compounds and proteins. Besides, the knowledge on its physiology, genetics and fermentations techniques is huge. Among all, *S. cerevisiae* has a food-grade status allowing its use in human nutrition and pharmaceuticals. Secondly, like *E. coli*, *S. cerevisiae* is easy to grow, manipulate and scale-up, it is well characterized and has been used to produce other polyketides. On the other hand, as an eukaryotic organism, it presents unique advantages over *E. coli* that facilitate the functional expression of plant derived genes. *S. cerevisiae* harbors post-translational machinery with intracellular compartments similar to plants. In addition, it has an endomembrane system and can functionally express cytochrome P450 containing enzymes such as C4H (Koopman et al., 2012). Another advantage is its excellent amenability to be manipulated through molecular and synthetic biology techniques, taking as example the development of new methods for rapid design and construction of large biochemical pathways like the "DNA assembler" (Shao et al. 2009; Yuan and Ching 2015). Glycosylation is the most abundant protein modification across all organisms (Larkin and Imperiali, 2011). On the other hand, *S. cerevisiae* has an extensive glycosylation level, referred as hyperglycosylation. The hyperglycosylation process may influence the solubility, folding and stability of foreign enzymes leading to its inactivation which could represent a problem for the heterologous production (Yin et al., 2007). In short, despite some possible difficulties, yeast seems to be the ideal host to produce curcuminoids. In the work of Beekwilder et al. (2006), resveratrol was produced with *p*-coumaric acid as precursor in engineered *E. coli* (16 mg/L) and in engineered *S. cerevisiae* (6 mg/L). These results do not imply that the engineered *E. coli* will produce larger amounts of products than the recombinant yeast as both growth conditions and strains could be further optimized. Flavonoids and stilbenoids have already been produced in engineered *S. cerevisiae* yielding positive results (Kim et al. 2005; Trantas et al. 2009; Zhang et al. 2006). In **Table 4** are presented some works on the heterologous production of plant phenolic compounds using yeast as the host system.

**Table 4**: Some works on the heterologous production of some phenolic plant compounds using *S. cerevisiae* as the host system. The concentrations are displayed in the same units that are present in the referred works.

| Genes used | Substrate (Concentration) | Production (Concentration) | Reference |
|---|---|---|---|
| *Rhodotorula glutinis* (PAL), *Helianthus tuberosus* (C4H, CPR) | L-phenylalanine (1 mM) | *p*-coumaric acid (498 μM) | (Vannelli et al., 2007) |
| *Populus trichocarpa* (4CL) *Vitis vinifera* (RS) | *p*-coumaric acid (10 mg/L) | Resveratrol (1.38 μg/L) | (Becker et al., 2003) |
| *Arabidopsis thaliana* (4CL1) *Arachis. hypogaea* (STS) | *p*-coumaric acid (16 mg/L) | Resveratrol (3.1 mg/L) | (Shin et al, 2011) |
| *R. toruloides* (PAL) *A. thaliana* (C4H and 4CL1) *A. hypogaea* (STS) | Tyrosine (2 mM) | Resveratrol (5.8 mg/L) | (Shin et al., 2012) |
| *Rhodosporidium toruloides* (PAL) *A. thaliana* (4CL) *Hypericum androsaemum* (CHS) | Tyrosine and phenylalanine (65 mg/L each) | Naringenin (7 mg/L) | (Jiang et al., 2005) |

## 1.5 Optimization of the curcuminoid biosynthetic pathway

### 1.5.1 From amino acids to coenzyme A esters

*S. cerevisiae* lacks the CoA esters, i.e. the starter substrates needed to produce curcumin. In addition, phenylalanine and tyrosine, naturally produced amino acids, which are converted into phenylpropanoic acids, and then in CoA-esters, are not produced in sufficient amounts representing a limiting step (Jiang et al., 2005; Kang et al., 2012). The aromatic amino acids biosynthesis is a branched pathway regulated at transcriptional, translational and allosteric levels. At allosteric level, the effector molecules are the end products, for example the reaction catalysed by chorismate mutase (the last common step in tyrosine and phenylalanine biosynthesis) is feedback-inhibited by tyrosine, but curiously no effect of phenylalanine is known (Schmidheini et al., 1990). Despite amino acids or phenylpropanoids can be supplemented to the culture medium, the use of tyrosine/phenylalanine overproduction strains represent the cheapest solution for this problem and has already been used. For instance, Koopman et al. (2012) using a mutated strain of *S. cerevisiae* with feedback

inhibition-resistance to produce naringenin, obtained larger amounts of the product comparatively with the wild type strain. Supplementing the media with curcumin precursors, ferulic acid or phenylalanine, makes the process expensive for industrial applications, therefore the development of strains capable of converting glucose or other simple carbon sources to curcumin represents also an important strategy for engineering processes. Li et al., (2015) constructed a pathway for *de novo* biosynthesis of resveratrol from glucose generating 2.73 + 0.05 mg/L of the polyphenol. In another experiment, to improve the flux towards aromatic amino acids, the researchers developed a feedback-inhibition resistant strain overexpressing chorismate mutase and 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase resulting in 4.85 + 0.31 mg/L resveratrol produced from 30 g/L glucose. Comparing these results with the resveratrol production yields shown in **Table 4**, the *de novo* production seems to be a very attractive approach for the polyphenol production in yeast mainly for industrial applications.

PALs from some plants, like *Zea mays* also have tyrosine ammonia lyase (TAL) activity, converting tyrosine to *p*-coumaric acid, the substrate of 4CL. PAL enzymes that allow *p*-coumaric acid to be produced without requiring the C4H step represent an advantage to prokaryotes because C4H is a membrane bound cytochrome P450-dependent (Rösler et al., 1997). Some authors reported their findings in bacterial TALs. TALs from *Rhodobacter spaheroids* and *Rhodobacter capsulatus* showed a clear preference for tyrosine instead of phenylalanine (Berner et al., 2006). In some works, using *S. cerevisiae* as the host for the production of stilbenoids and flavonoids, PAL and also C4H (this enzyme can be expressed in yeast due to the cytochrome P450 reductase activity) were used. C4H from *A. thaliana* and *Glycine max* were successfully cloned and expressed in *S. cerevisiae*, but its activity remains a rate limiting step since the endogenous cytochrome P450 reductase activity is not enough to support its expression (Trantas et al., 2009; Yan et al., 2005).

### 1.5.2 4-Coumarate-CoA reaction

The 4CL enzymes family catalyses the conversion of *p*-coumaric acid, ferulic acid and caffeic acid to the respective CoA esters. 4CL has been shown to occur in the form of multiple isoenzymes with distinct substrate affinities (Ehlting et al., 1999). Much of the work in the heterologous production of flavonoids and stilbenoids in *S. cerevisiae* used 4CL from *G. max* or *A. thaliana* (**Table 5** and **Table 6**). This seems to be the better approach since these organisms are the best characterized in the plant kingdom. For example, *A. thaliana* possesses four isoforms of 4CL (At4CL1, At4CL2, At4CL3 and At4CL4). From the results of expression patterns and phylogenetic similarity, At4CL3 is suggested to play a role in flavonoid biosynthesis, while At4CL1 and At4CL2 are presumed to be involved in lignin production. At4CL4 exhibits a substrate preference to ferulic acid and sinapic acid over *p*-coumaric acid (Hamberger and Hahlbrock, 2004; Saito et al., 2013) which may be an advantageous strategy for the heterologous production of curcumin. Li et al. (2015) also tested if the use of different *A. thaliana* 4CL isoforms (4CL1 and 4CL2) would have an impact on the production of resveratrol by the engineered yeasts. The results confirmed that 4CL1 consistently resulted in slightly higher resveratrol titters than 4CL2 confirming the high activity of 4CL1 when *p*-coumaric acid is the substrate which agrees with the results obtained by Ehlting et al. (1999).

**Table 5**: Kinetic parameters of the three isoforms of 4CL from *Glycine max*

| Organism | Abbreviation | Substract | $K_m$ (mM) | Reference |
|---|---|---|---|---|
| *G. Max* | Gm4CL1 | *p*-coumaric acid | 0.05 | (Lindermayr et al., 2003) |
| | | caffeic acid | 0.081 | |
| | | ferulic acid | 0.004 | |
| | Gm4CL2 | *p*-coumaric acid | 0.042 | |
| | | caffeic acid | 0.013 | |
| | | ferulic acid | 0.14 | |
| | Gm4CL3 | *p*-coumaric acid | 0.009 | |
| | | caffeic acid | 0.05 | |
| | | ferulic acid | 3.1 | |

**Table 6**: Kinetic parameters of the three isoforms of 4CL from *Arabidopsis thaliana*

| Organism | Abbreviation | Substract | $K_m$ (mM) | Reference |
|---|---|---|---|---|
| *A. thaliana* | At4CL1 | *p*-coumaric acid | 0.038 | (Ehlting et al., 1999; Hamberger & Hahlbrock, 2004) |
| | | caffeic acid | 0.011 | |
| | | ferulic acid | 0.199 | |
| | At4CL2 | *p*-coumaric acid | 0.252 | |
| | | caffeic acid | 0.020 | |
| | | ferulic acid | No cat. | |
| | At4CL3 | *p*-coumaric acid | 0.023 | |
| | | caffeic acid | 0.374 | |
| | | ferulic acid | 0.166 | |
| | At4CL4 | *p*-coumaric acid | 0.432 | |
| | | caffeic acid | 0.186 | |
| | | ferulic acid | 0.026 | |

Jiang et al., (2005) designed an engineered *S. cerevisiae* for the production of flavonoids. For this purpose, the researchers cloned PAL from *Rhodosporidium toruloides* (RtPAL) (this PAL also shows TAL activity), 4CL from *A. thaliana* and chalcone synthase (CHS) from *Hypericum androsaemum* in a *S. cerevisiae pad1* knockout strain. This gene encodes for phenylacrylic acid decarboxylase (PAD), an enzyme responsible for the reduction of cinnamic acid and *p*-coumaric acid. Using this engineered strain 5.8 mg/L of naringerin (flavonoid derived from tyrosine) and traces of pinocembrin (flavonoid derived from phenylalanine) were produced. Besides, to ensure the activity of each enzymatic step, the authors constructed yeast strains expressing each gene. To analyse the RtPAL activity, the concentrations of cinnamic acid and *p*-coumaric acid were measured in yeast expressing only RtPAL after induction. In order to analyse the At4CL activity, the crude protein mixture was extracted and the production of *p*-coumaroyl-CoA was followed by ultraviolet-visible spectroscopy. *p*-Coumaroyl-CoA was produced in yeasts transformed with At4CL and RtPAL and in yeasts transformed only with At4CL. Besides naringenin and pinocembrin, other flavonoids by-products were also quantified. The researchers concluded that the At4CL also accepts cinnamic acid but with low efficiency due to the lower level production of pinocembrin. The lower level of flavonoids production derived from tyrosine was mainly due to the low level of *p*-coumaric acid production which led the authors to believe that there could be other

enzymes in addition to PAD capable of degrading the *p*-coumaric acid. Since *A. thaliana* C4H was previously successfully expressed in *S. cerevisiae* (Pompon et al., 1996), the researchers hypothesized that the introduction of C4H would compete with PAD and/or the unknown endogenous yeast enzymes that degrade cinnamic acid and redirect the carbon flux to *p*-coumaric acid, thus enhancing the production of naringenin.

The choice of the right enzymes combinations is a key step to produce heterologous compounds like curcumin in yeast. The utilization of enzymes with multiple substrate affinities like 4CL and CURS, enzymes that reduce the number of reactions required such as TAL, as well as the deletion or overexpression of genes that may hinder the production process represent all strategies of optimization at the genetic level which should be considered.

### 1.5.3 Ferulic acid availability

Ferulic acid is the precursor of curcumin. In *S. cerevisiae*, ferulic acid can be decarboxylated and converted in 2-methoxy-4-vinylphenol by ferulic acid decarboxylase (FDC) as a detoxification process (Huang et al., 1993; Mukai et al., 2010). FDC is encoded by *fdc1* and the utilization of a strain with *fdc1* knockout could represent an optimization method for the production of curcumin in *S. cerevisiae*. The development of knockout strains has already proved to be useful in the synthesis of polyphenols in *S. cerevisiae*. Shin et al, (2011) produced resveratrol using a *pad1* knockout *S. cerevisiae* strain. The deletion of PAD1 gene prevented the utilization of *p*-coumaric acid by yeast. Nevertheless, the development of a *fdc1* knockout strain may be an interesting strategy because a possible ferulic acid consumption could be prevented and it can be all used for curcumin synthesis.

### 1.5.4 Synthetic biology techniques

For metabolic engineering purposes, linking genes together to generate a functional fusion protein offers an attractive strategy for increasing product yields. The use of fusion proteins also reduces the number of vectors in a heterologous expression system, which simplifies the reconstitution of metabolic pathways. In addition, protein-protein interactions

may increase metabolic efficiency either by channelling intermediates between enzymes or by locating two active sites in a close proximity. This hypothesis was tested by Zhang et al., (2006). The authors constructed a translational fusion protein of 4CL from *A. thaliana* and stilbene synthase (STS) from *Vitis vinifera* (4CL::STS) for the resveratrol biosynthesis in transformed *S. cerevisiae*. The fusion protein consisted of the 4CL with its stop codon replaced by a three-amino acid linker, followed by STS. The yeasts transformed with 4CL::STS fusion protein increased resveratrol production by up to 15-fold compared to transformants coexpressing 4CL and STS. More recently, Wang and Yu (2012) used a synthetic scaffolds strategy to produce resveratrol in yeast with the same enzymes and observed a 5.0-fold improvement over the non-scaffold control, and a 2.7-fold increase over the previous reported with fusion protein. This work demonstrated that the synthetic scaffolds can be used for the optimization of engineered metabolic pathways. Zhang et al., (2016) already produced an unnatural DCS and CURS fusion protein for heterologous production of curcuminoids in *E. coli*. The researchers used DCS from *C. longa* and the recently identified by CURS from *Z. officinale*. The yield of curcuminoids produced by DCS::CURS fusion protein was higher relatively to that produced by *E. coli* transformed with DCS and CURS. This was the first study in which fusion proteins were used to produce curcuminoids.

Codon optimization must be a strategy to consider, due to the existence of rare codons in heterologous genes, the expression of those genes can lead to translational errors, frameshifting events, stalling or premature translational termination, especially when transcripts containing rare codons accumulate in large quantities (McNulty et al., 2003). Codon optimization has been shown to improve activities of pathway enzymes relative to the ones from native genes and it is now a commonly used technique in the construction of heterologous pathways (Redding-Johanson et al., 2011). Wang et al. (2011) showed that codon optimization in *S. cerevisiae* drastically improved the production of resveratrol *in vivo*. In this work it was also demonstrated that expression of a non-specific transporter was important to increase the resveratrol productivity. Curcumin and resveratrol, like other polyphenols, accumulate in plant vacuoles (Dixon and Paiva, 1995) and the transportation of these compounds from vacuoles is not fully understood. Wang et al. (2011) also expressed the arabinose-H+ transport protein (AraE) in the engineered yeast. The transporter greatly improved the resveratrol synthesis indicating that AraE may increase resveratrol permeability

through lipid membranes when resveratrol levels are elevated over a threshold. Maybe a similar strategy could be useful to increase the curcuminoids production.

### 1.5.5 Malonyl-CoA availability

In the curcuminoids production it is necessary one molecule of malonyl-CoA to obtain the final product. However, malonyl-CoA inside the cell is at low concentrations which could represent a limiting step in the heterologous production of curcuminoids. Malonyl-CoA is naturally synthesized by ACC (catalyses the carboxylation of acetyl-CoA to malonyl-CoA) in microorganisms but it is used for the production of fatty acids and phospholipids, leaving only a very limited amount available for the production of secondary metabolites (Takamura and Nomura, 1988). The engineering of the microbial host to achieve metabolic balance between the need for malonyl-CoA for growth and secondary metabolite production is essential. In bacteria and plant cells, malonyl-CoA can be generated through the condensation of malonic acid. Studies on overexpression of a plant malonyl-CoA synthase in *S. cerevisiae* resulted in a 1.6 fold increase in lipid content, which suggested the increase of the malonyl-CoA pool (Tang et al., 2015). Engineering microorganisms with ACC genes from other organisms or changing the promoter of ACC to a strong constitutive promoter are strategies that have been used to develop engineered strains overproducing ACC (Miyahisa et al., 2005; Wattanachaisaereekul et al., 2008). ACC in yeast is subject to inactivation via phosphorylation by a kinase, called sucrose non-fermenting protein kinase (SFN1). Studies demonstrate that site mutations in ACC could enhance the supply of malonyl-CoA by inhibiting phosphorylation of ACC (Shi et al., 2014). Since ACC belongs to the family of biotin dependent carboxylases, the efficiency of ACC biotinylation controls its activity, and it has been reported that the reduced level of this vitamin affects the synthesis of fatty acids suggesting its role in malonyl-CoA production (Suomalainen and Keränen, 1963). For instance, to increase the pool of malonyl-CoA for resveratrol production in an engineered yeast, Shin et al. (2012) elevated the expression level of ACC1 transcript by replacing the native promoter for GAL promoter. The overexpression of the ACC1 gene resulted in 1.3-fold increase in resveratrol concentration.

## 1.6 Heterologous production in *Saccharomyces cerevisiae*

### 1.6.1 Culture media

The use of a proper culture medium may be the key point to obtain large quantities of the desired product. The optimization of the fermentation conditions should be an aspect to consider to produce curcumin in yeast. Sydor et al. (2010) studied the resveratrol synthesis from *p*-coumaric acid in *S. cerevisiae* expressing the At4CL1 and STS from *V. vinifera* growing in rich (YPD) or synthetic medium (SD) supplemented with 5 mM of p-coumaric acid. Yeasts grown in SD medium produced 6 mg/L of resveratrol, while in YPD medium 391 mg/L were produced. Therefore, the authors concluded that resveratrol productivity can be increased by fermentation in YPD instead of SD medium because higher amounts of biomass are obtained. Curcuminoids have never been produced in *S. cerevisiae* so there is no specific information on the culture media that can favour its production. The YPD medium is generally used in the production of other polyketides. However, the use of rich media has the disadvantage of not allowing the selection of transformants when auxotrophic markers are used. Rice bran pitch, an industrial waste obtained from rice bran in the production of rice oil and rich in ferulic acid, was used by Katsuyama et al. (2008) to produce curcumin in *E. coli* yielding 57± 21 mg/L of curcumin.

### 1.6.2 Vectors and promoters

In order to increase the productivity many factors must be considered including optimizing the pathway flux, using gene deletion or overexpression, as mentioned, as well as reducing the toxic intermediates and balancing the stress on the cell. Thereupon, the choice of the right expression vector is a key step in pathway engineering. Most expression plasmids allow the expression of only one gene. However, for the expression of an entire metabolic pathway, it is desirable to express more than one gene per plasmid unit. Different expression systems, such as yeast integrative plasmids (YIps) for integration of the desired gene into the yeast genome or yeast episomal plasmids (YEps) for high copy number expression, have been

designed and developed. The use of plasmids imposes a metabolic burden on the host that usually reduces the growth rate of the cell. The two-step fermentation is a commonly used strategy to compensate this metabolic burden. The vector copy number is very important because not always a high copy number is desired. High copy plasmids rely the 2µ origin of replication which allows about 30 copies per cell (Mumberg et al., 1995). This kind of plasmid allows a strong gene expression which can cause an excessive burden resulting in instability of the construct. The low copy plasmids have an autonomously replication sequence paired with centromere to allow stable maintenance at very low copy (Parent et al., 1985). Low copy plasmids provide a more stable expression platform, but with lower gene expression. However, when YEps is used a selective pressure is required. YIps provide the most stable platform and does not require continuous selective pressure but the expression levels are lower. Jordan and Nash (2015) studied the plasmid loss rate in *S. cerevisiae* harbouring YEpd in the absence of selective pressure. The authors concluded that yeast maintain the plasmids much longer than expected suggesting that growth in non-selective medium may be a viable method. Usually, the selective pressure is exerted by an auxotrophic marker which makes impossible the use of rich media. Sydor et al. (2010), mentioned above, used 2µ plasmids with an antibiotic resistance marker making possible the use of rich media.

The choice of the promoter controlling the expression of the pathway genes is also very important. *S. cerevisiae* has a set of well characterized promoters with different expression levels (Partow et al., 2010). These promoters can be grouped by the type of control, i.e. constitutive and inducible. Inducible promoters have the advantage of being able to control enzyme levels but have the disadvantage that the inducer molecules for these systems have known pleiotropic effects as also the cells may consume the inducers, galactose for instance, decreasing the gene expression (Mumberg et al., 1995).

## 1.7 Curcumin extraction, quantification and characterization

The extraction is an essential step to obtain compounds produced in heterologous organisms, because if the extraction process is not efficient the whole process is compromised. Curcuminoids are purified by solvent extraction in turmeric and this method was also used in the works in which the curcuminoids were produced in *E. coli*. After the

culture is adjusted to pH 3.0 with HCl, the extraction of curcuminoids is proceeded with equal volume of ethyl acetate and then concentrated by evaporation (Katsuyama et al., 2007). In a study performed by Kulkarni et al. (2012), several solvents with varying polarities were evaluated to extract curcuminoids in turmeric. The extraction with methanol yielded the greater amounts of curcuminoids followed by extraction with sodium acetate. The lysis of the cells before extraction was found to increase the amount of the extracted product, since the produced curcuminoids can remain inside the cells.

Many methods for the quantification of the curcuminoids have been reported including high-performance thin-layer chromatography (HPTLC) (Ansari et al., 2005), high-performance liquid chromatography (HPLC) (Jayaprakasha et al., 2002), and mass spectrometry (MS) (Inoue et al., 2007). These methods allow both the quantification and characterization of the various curcuminoids.

# 2 OBJECTIVES

The main goal of this work was to design, construct and validate a biosynthetic pathway composed by genes from different plants to produce curcumin from ferulic acid in an engineered *S. cerevisiae*. Other specific aims included:

- Evaluation of *S. cerevisiae* behaviour in the presence of different concentrations of ferulic acid;
- Design, construction and validation of three different curcumin biosynthetic pathways using molecular and synthetic biology techniques;
- Evaluation of the efficiency of diverse curcumin extraction methods;
- Development of a *fdc1* gene knockout *S. cerevisiae* strain using CRISPR-Cas9 technology;
- Evaluation of *S. cerevisiae* mutant in curcumin production.

# 3 MATERIALS AND METHODS

## 3.1 Strains, plasmids and genes

*E. coli* NZY5α (NZYTech) competent cells were used for molecular cloning, vector propagation and storage. *S. cerevisiae* CEN.PK2-1C and *S. cerevisiae* Y02123 were used as hosts for genes expression and curcumin production (relevant genotype information provided in Annex A). Moreover, *S. cerevisiae* CEN.PK2-1C was also used for curcumin production after knockout of *fdc1* gene (**Section 3.8**).

The vector used for the pathway construction was pSP-GM1, kindly provided by Jens Nielsen, Chalmers University of Technology, Sweden (Addgene plasmid # 64739) (Chen et al., 2012). This shuttle vector has 7404 bp, possesses the ampicillin resistance gene (for selection in *E. coli*), the URA3 gene (for selection in *S. cerevisiae*) and two *S. cerevisiae* constitutive promoters (TEF1 (3' -> 5') and PGK1 (5' -> 3') with the respective terminators (ADH1 and CYC1). Another vector herein used was the pCRCT (Addgene plasmid # 60621) (Bao et al., 2015) for CRISP-Cas9 method. This plasmid holds the ampicillin resistance gene and the URA3 gene, and also possesses the LacZ gene for blue and white screening and all the machinery necessary for CRISPR-Cas9 namely the iCas9 gene and the tracrRNA. The maps of the commercial vectors are available in **Figure B1** and **Figure B2** in **Annex B**. All the plasmids used and constructed in this work are displayed in **Table B1** in **Annex B**.

The genes *At*4CL, *Le*4CL and CUS and the TDH3 promoter were obtained from plasmids previously constructed (Rodrigues, 2014; Rodrigues et al., 2015). DCS and CURS1 genes codon-optimized for *S. cerevisiae* were synthetized and cloned into pNZY29 vector by NZYTech. These plasmids were transformed into *E. coli* NZY5α cells for propagation and storage. The codon optimized sequences are provided in **Annex C**. The double strand DNA fragment used for *fdc1* knockout using CRISPR-Cas9 (gBlock) was synthetized by Integrated DNA Technologies (IDT).

## 3.2 Chemicals, culture media and microbial growth conditions

Standard reagents were used to prepare the culture mediums: LB broth and SOC broth (NZYTech); YPD media (3.3% (p/v) Yeast extract (Panreac Applichem), 2% (p/v) peptone (Fluka), 2% (p/v) glucose (Acros Organics) and 0.7% (p/v) YNB minimal media without amino acids (Sigma) with also 2% (p/v) of glucose as carbon source. Ampicillin (AppliChem) was used at a final concentration of 100 µg/mL and the required amino acids (L-histidine and L-leucine (Fisher), L-tryptophan (Sigma) and L-methionine (AppliChem)) at a final concentration of 25 µg/mL. Uracil (URA) (Sigma) was used with a final concentration of 50 µg/mL. The agar plates contained 20 g/L of agar (Liofilmchem). Glycerol (Himedia) solution 70% (v/v) was used for culture storage.

*E. coli* cell cultures were cultivated at 37ºC and 200 rpm. Transformants were selected in LB solid medium supplemented with ampicillin. *S. cerevisiae* was grown at 30 ºC and 250 rpm, and the transformants were selected in YNB solid media supplemented with the required amino acids lacking uracil for selection (YNB -URA). For plasmid curing in yeast, YNB plasmid curing plates contained 5 g/L of 5-fluoroorotic acid (5-FOA) (Sigma).

For yeast transformation, 1 M lithium acetate (Sigma), 50% (p/v) polyethylene glycol 3000 (PEG 3000) (Sigma) and 10 mg/mL deoxyribonucleic acid low molecular weight from salmon sperm (ssDNA) (Sigma) solutions were prepared. Ferulic acid (Acros Organics) diluted in dimethyl sulfoxide (DMSO) (Fisher Scientific) was used as substrate for curcumin production. Curcumin (95 % purity) (Acros Organics), 2-Methoxy-4-vinylphenol (Alfa Aesar), methanol (Fisher Scientific), acetonitrile (Fisher Scientific), trifluoracetic acid (TFA) (Fluka), phosphoric acid (Sigma), ethanol (Fisher Scientific) and ethyl acetate (Fisher Scientific) were used for the curcumin extraction process and for high-performance liquid chromatography (HPLC) analysis.

## 3.3 Pathway and primer design

For curcumin production in yeast, three different pathways were designed using pSP-GM1 vector. In two of the pathways CUS gene from *O. sativa* was cloned with 4CL from distinct organisms (*A. thaliana* and *L. erythrozium*). In the third pathway, DCS and CURS1 codon optimized for *S. cerevisiae* and 4CL1 from *A. thaliana* were used.

The primer design considered the insertion of the same restriction sites used to cut the plasmid between the respective promoter and terminator as well as the insertion of a ribosome recognition site (5'- AAAAAA - 3') before the starting codon of each gene. Two distinct enzymes which originate cohesive ends were used for a single insert to ensure the insertion of the gene with the right orientation. All the primers and plasmids are displayed in **Table D1** (**Annex D**) and **Table B1** (**Annex B**), respectively. The primers used for colony PCR and sequencing are described in **Table D2**.

Since pSP-GM1 only has two multiple cloning sites, and for the third pathway it is necessary to insert three genes, the TDH3 promoter and synthetic terminator 27 were also cloned to express a third gene (CURS1) in pSP-GM1. For that purpose, the promoter TDH3 was first inserted in pSP-GM1_DCS_4CL forming the plasmid pSP-GM1_DCS_4CL_TDH3. Note that, TDH3_REV primer, besides the AvrII sequence, possesses also the SphI sequence which allowed to insert the SphI restriction site in the plasmid pSP-GM1_DCS_4CL_TDH3 right after the TDH3 sequence. Afterwards, the SphI site was used to clone the CURS1 gene. Besides, the reverse primer for CURS1 possessed the sequence of the synthetic terminator 27 allowing the construction of a cassette composed by the gene and the regulatory region. This strategy can be visualized in **Figure 3**.

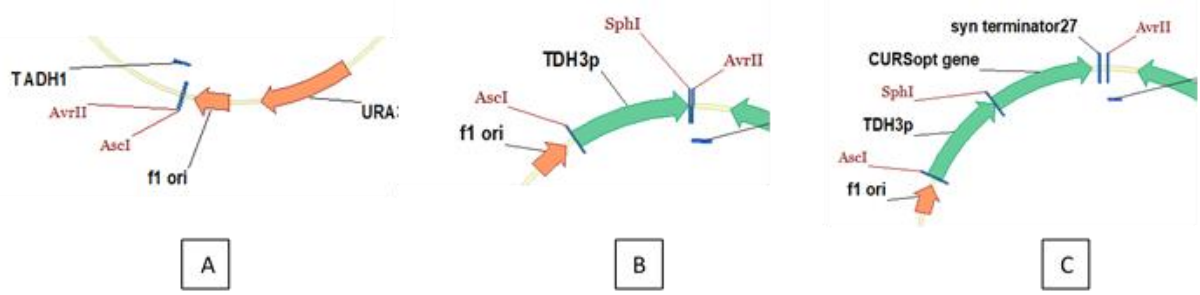



**Figure 3: Strategy used to insert the third gene in pSP-GM1_DCS_At4CL_CURS1**: **A**: pSP-GM1_DCS_At4CL with AscI and AvrII sites highlighted; **B**: TDH3 promoter and SphI restriction site insertion forming pSP-GM1_DCS_At4CL_TDH3; **C**: CURS1 gene and synthetic terminator 27 insertion forming pSP-GM1_DCS_At4CL_CURS1.

## 3.4 Plasmid construction

Plasmid DNA (pAt4CL, pLe4CL, pDCS, pCURS, pCUS, pSP-GM1 and pCRCT) was extracted using NucleoSpin® Plasmid extraction kit (Machinery-Nagel) from an overnight growth of *E. coli* culture.

The genes were PCR-amplified using the homonymous primers and Phusion DNA Polymerase (Thermo) and homonymous plasmids as DNA template (PCR conditions displayed in **Table 7**) and purified from agarose gel using NucleoSpin® Gel and PCR Clean-up (Machinery-Nagel). DNA Electrophoresis was performed with a 0.7 % (p/v) agarose (Fisher Scientific), during 30 min at 90 V. A loading dye containing bromophenol blue and glycerol was loaded with the sample and NZYDNA ladder III (NZYTech) was used as a marker. Thiazole orange (Sigma) was used as DNA dye. The bands were visualized using ChemiDoc Imaging System (Bio-Rad). The plasmid DNA and genes were quantified by NanoDrop 1000 (Thermo).

**Table 7**: PCR conditions used to amplify the genes and TDH3 promoter using Phusion High-Fidelity DNA polymerase

| Cycling step | | At4CL | Le4CL | CUS | DCS | At4CL_2 | TDH3 | CURS1 |
|---|---|---|---|---|---|---|---|---|
| Initial denaturation | | 98 °C, 30 s | | | | | | |
| Denaturation | 30x | 98 °C, 5 s | | | | | | |
| Annealing | | 63 °C, 20 s | 63 °C, 20 s | 66 °C, 20 s | 56 °C, 20 s | 62 °C, 20 s | 58 °C, 20 s | 58 °C, 20 s |
| Extension | | 72 °C, 52 s | 72 °C, 58 s | 72 °C, 37 s | 72 °C, 36 s | 72 °C, 52 s | 72 °C, 31 s | 72 °C, 38 s |
| Final extension | | 72 °C, 5 min | | | | | | |

Afterwards, the plasmid DNA and the genes were digested with the respective restriction endonucleases (Thermo) for 3 h at 37°C and purified using the extraction kit. The plasmid DNA was first run in agarose gel after digestion and then purified. Then, the fragments were ligated using T4 DNA ligase (Thermo), at room temperature for 1 h. *E. coli* NZY5α competent cells were chemically transformed by heat-shock method, according to the instructions provided by NZYtech. The constructed plasmids were verified by colony PCR using

the specific primers for pSP-GM1 and KAPA *Taq* DNA polymerase (KAPA Biosystems). Next, the plasmids were digested and further confirmed by sequencing (GATC Biotech, Konstanz, Germany) (Information about colony PCR conditions present in **Table E1** and **Table E2** in **Annex E**). In all cases the engineered strains were cryopreserved at -80ºC in glycerol.

## 3.5 Yeast transformation

All the constructed plasmids were first transformed in *E. coli* NZY5α cells using heat-shock method to ensure the plasmid propagation as already mentioned. After that, plasmid DNA was extracted using the purification kit from an overnight culture grown in LB supplemented with ampicillin. Next, the vectors pSP-GM1_Le4CL_CUS, pSP-GM1_At4CL_CUS and pSP-GM1_DCS_At4CL_CURS were transformed into the yeast strains using lithium acetate/single-stranded carrier DNA/PEG method (Gietz, 2014). Some modifications were added to the method in order to improve the transformation efficiency: the transformation mix was maintained in ice for 30 min before the heat-shock and after the heat-shock the cells were inoculated with 1 mL YPD and maintained at 30ºC, 200 rpm during 1 h. The transformants were plated in selective medium (YNB-URA) and the plates were maintained at 30ºC during approximately 3-4 days. Afterwards, the colonies were inoculated in 5 mL of liquid selective medium and grown overnight at 30ºC, 200 rpm. Then, 2 mL of the culture was stored at -80ºC in glycerol and YNB -URA agar plates were prepared using the streak technique allowing to obtain multiple colonies from a single transformant for further utilization.

## 3.6 Batch fermentation

A single colony was used to prepare a 5 mL pre-culture, in 50 mL falcon tubes. Rich YPD medium was used for wild-type strains and synthetic defined YNB medium without uracil (to ensure the selective pressure) was used for the engineered strains. When the pre-culture reached an $OD_{600nm}$ > 1, 1 mL of the pre-culture was used to inoculate 50 mL of media in 250 mL shake flasks. The optical density was measured using spectrophotometer UV-3100OC (VWR).

### 3.6.1 Dry weight calibration

*S. cerevisiae* CEN.PK2-1C was grown in 50 mL YNB broth. The fermentation was maintained until a constant $OD_{600nm}$ was reached. At this point, successive dilutions of the sample were measured. Five membranes (porosity 0.2 µm) (GE Healthcare) were weighed. Three membranes were used to filtrate 5 mL of culture, followed with a wash with 5 mL of water. The other two membranes were used as control being only filtered 5 mL of water. The filters were dried in the microwave for 10 min at 150 W and placed in the drying oven at 55$^{o}$C for more 15 min. After that, the filters were weighed and the dry weight biomass calculated by the difference in weight before and after fermentation. This process allowed to construct the calibration curve of biomass concentration (g/L) *versus* $OD_{600nm}$ present in **Figure F1** in **Annex F**.

### 3.6.2 Ferulic acid toxicity assay

To evaluate if ferulic acid influences yeast growth a toxicity assay was performed. For this purpose, four 250 mL flasks with 50 mL YNB media (2% (w/v) glucose) supplemented with the required nutrients were inoculated with 1 mL of the grown pre-culture of *S. cerevisiae* CEN.PK2-1C wild-type. The fermentation was carried at 30 $^{o}$C and 250 rpm. Samples were taken at certain time points: 6h, 24h and 48h to measure the $OD_{600nm}$ and for further HPLC analysis. When the $OD_{600nm}$ reached a value of 0.2, ferulic acid diluted in DMSO was added to the culture media at different final concentrations: 0.5 mM, 1 mM, 2mM and 5mM. The fermentation process was maintained during 48 h.

### 3.6.3 Curcumin production

Yeast strains transformed with pSP-GM1_At4CL_CUS, pSP-GM1_Le4CL_CUS, pSPGM1_DCS_4CL_CURS1 were tested for curcumin productivity. For this purpose, 1 mL of the pre-inoculum (2% of the final volume) was inoculated in 50 mL of YNB -URA with 2% (w/v) glucose in 250 mL shake flasks. A solution of ferulic acid diluted in DMSO was prepared and

different substrate concentrations were added to the media 0.08 mM (16 mg/L), 0.4 mM (80 mg/L), 0.8 mM (160 mg/L) and 2 mM (388.3 mg/L). The fermentation process was accompanied for 6 days at 30ºC and 250 rpm and 2 mL samples were taken every 24 h to measure the $OD_{600nm}$. The curcumin production was confirmed by the yellow colour formation in the culture media. A representative scheme of the fermentation process is shown in the **Figure 4**.

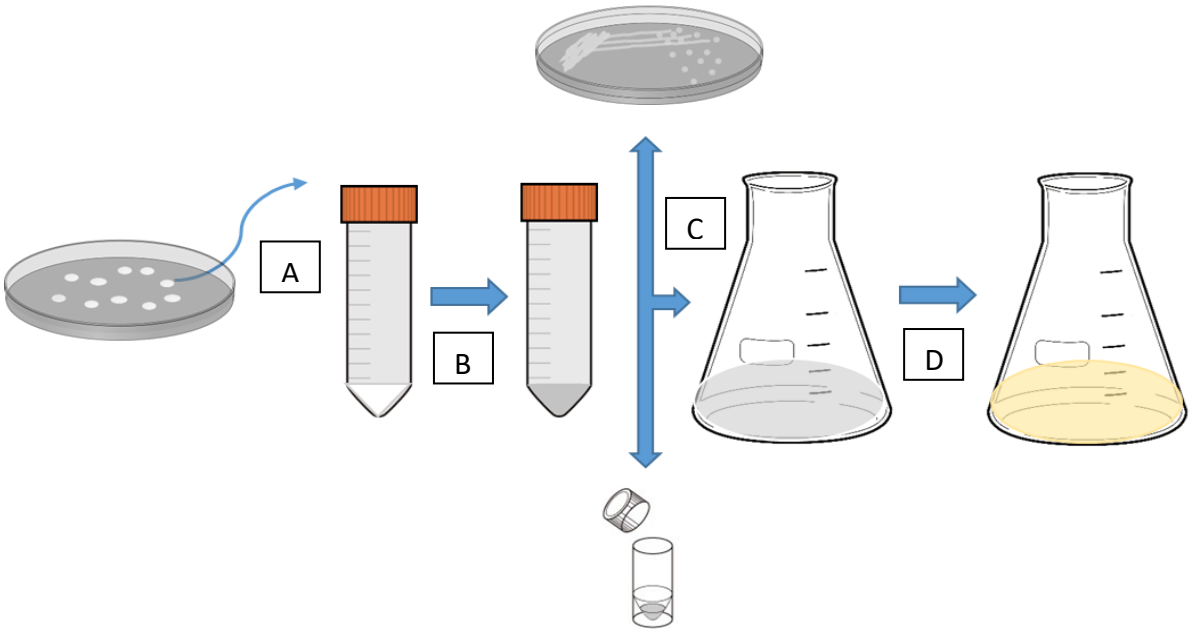



**Figure 4: General scheme of the fermentation process**: **A**: A single colony was picked from a YNB- URA transformation plate to prepare a 5 mL pre-culture in YNB selective media; **B**: Pre-inoculum growth at 30 ºC until an $OD_{600\ nm}$ >1 was reached; **C**: 1 mL of the pre-inoculum was inoculated in 50 mL selective media, 2 mL cryopreserved at -80 ºC and streak plate to isolate single colonies; **D**: The fermentation was carried at 30 ºC, 250 rpm in the presence of ferulic acid. The yellow colour formation confirmed the curcumin production. Colours are not representative.

## 3.7 Curcumin extraction

To extract curcumin, three different methods were tested. In the first one, yellow cultures (50 mL) were spun down (13000 g for 2 min) and the supernatant (culture media) was removed. The yellow biomass, suggesting that curcumin remains inside the cells, was re-suspended in 1 mL of methanol and vortexed vigorously for 2 min. After that, the liquid phase was separated from the biomass and placed in a clean 2 mL centrifuge tube and the samples were concentrated by evaporation of the methanol in a fume hood.

The extraction was also tested using ethanol 70 % (v/v) to break the cells. For that, 8 mL of the culture media was removed, the biomass was suspended in 1 mL of the ethanol solution

and vortexed during 10 min. Afterwards, ethanol was evaporated and curcumin was extracted from the remaining water with 500 µL of ethyl acetate and concentrated by evaporation.
In the third method glass beads were used to break the cells. For that, FastPrep®-24 (MP Biomedics) cell disruptor was used. Lysis tubes with 1 mL of culture together with glass beads (diameter: 0.5 mm) were prepared. The program was set for 60 m/s during 1 min followed by 1 min on ice. The process was repeated three times. Next, the liquid phase and the glass beads were separated by centrifugation. Curcumin was extracted with 500 µL ethyl acetate from the liquid phase and concentrated by evaporation.

Afterwards, in the three extraction approaches, the dried curcumin was re-suspended in 300 µL of acetonitrile for further HPLC analysis.

### 3.7.1 HPLC analysis

HPLC was used to quantify the produced curcumin as well as to quantify the consumption of ferulic acid and the formation of 2-methoxy-4-vinylphenol during the fermentation process. The chromatographic system was composed by Shimadzu Nexera-X2 (Shimadzu Corporation, Kyoto, Japan) (CBM-20A system controller, LC-30AD pump unit, DGU-20A 5R degasser unit, SPD-M20A detector unit, SIL-30AC autosampler unit, CTO-20AC column oven) system and the column was a Platinum 100 C18 EPS (3 µm, 150 mm × 4.6 mm) (Dr. Maisch). For curcumin detection and quantification mobile phase A was composed of water with 0.1% of TFA and the mobile phase B was composed of acetonitrile. A gradient of 40-43% acetonitrile for 20 min was used at a flow-rate of 1 mL/min and curcumin was detected at 425 nm and in a retention time of 15.5 min. For ferulic acid and 2-methoxy-4-vinylphenol quantification, mobile phase A was composed of water with 1% of phosphoric acid and the mobile phase B was methanol with 1% phosphoric acid. A ratio 60:40 (water:methanol) was used at a flow-rate of 1 mL/min for 10 min. Ferulic acid and 2-methoxy-4-vinylphenol were detected at 310 nm and 240 nm and in a retention time of 2.4 min and 6.7 min, respectively.

## 3.8 Gene deletion

### 3.8.1 CRISPR-Cas9 target site selection and sequence design

The sequence that directs the Cas9 protein to cleave the target DNA sequence (*fdc1* gene) was ordered as a gBlock (IDT) as described in **Figure 5**. In this case, CRISPR technology was used to cause an 8 bp deletion in *fdc1* gene. The 8 bp deletion causes an alteration in the gene open reading frame putting a stop codon in frame resulting in no enzyme expression.

The first step in the design of this sequence was to choose a PAM (protospacer adjacent motif) site in *fdc1* gene (see Annex G). PAM is a DNA sequence recognized by Cas9 directly downstream of the target sequence and is composed of two guanines (5'-NGG-3'). In this case, PAM was selected between bases 109 and 111 of the *fdc1* gene. After this it was necessary to identify the protospacer (guide sequence), that is the part of the sgRNA sequence that is complementary to the target sequence. This sequence possesses the first three bases of the PAM site and the previous 17 bp in *fdc1* gene. Following, the homology zones, correspondent to the 50 bp upstream and downstream of the 8 bp deletion.

Next, to design the DNA fragment for CRISPR-Cas9 (gBlock) the identified sequences were organized in the following order: 50 bp upstream homology, 50 bp downstream homology and guide sequence (**Figure 5**). The BsaI recognition site was also inserted in both ends of the gBlock.

**GGTCTCCCCAAAGATGAAGATGACTTAATCGAAATTACCGAAGAGATTGATCCAAAT**
**CTCGACAATTATGAGGAAGGCCTATGAATCCCACTTACCAGCCCCGTTATTTAAAATT**
**GATCCAAATCTCGAAGTTAGAGCGAGACC**

**Figure 5: Synthetized gBlock**: Yellow: BsaI recognition site; White: pCRCT complementarity; Light blue: 50 bp upstream homology zone; Gray: 50 bp downstream homology zone; Green: guide sequence.

This enzyme does not cut the DNA at the recognition site and to ensure that the digested gBlock ligates with the plasmid, the same adjacent bases to the BsaI sites in the plasmid were added in the gBlock. This also ensures the insertion of the gBlock with the right

orientation. It was important to include the PAM sequence in the 8 bp deletion (see **Annex G**) to prevent continuous recognition and cleavage by CRISPR-Cas9 after the recombination event. A scheme showing *fdc1* gene after CRISPR-Cas9 method is present in **Figure 6**.

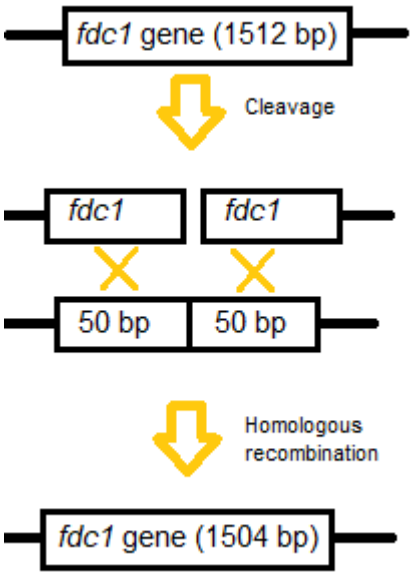



**Figure 6***:* Scheme showing *fdc1* after Cas9 cleavage and homologous recombination.

### 3.8.2 Golden Gate Assembly and CRISPR-Cas9 method

To knockout the *fdc1* gene from *S. cerevisiae* CEN.PK2-1C CRISPR-Cas9 technology was used. For this purpose, the synthetized gBlock was inserted into pCRCT vector using Golden Gate Assembly technique (Engler et al., 2008). First, a ligation mix of 10 µL was prepared containing 2.5 µL BsaI (Thermo), 0.45 µL T4 ligase (Thermo), 1 µL T4 ligase buffer, 50 ng of plasmid and the 50 ng gBlock. The mix was maintained at 37ºC during 30 min followed by 5 min at 50ºC and then 5 min at 80ºC. The incubation at 50ºC was performed to re-digest any plasmid that might still contain a BsaI restriction site, while the 80ºC incubation aimed the inactivation of both restriction enzyme and ligase. Next, the ligation mix was transformed into *E. coli* NZY5α cells and the transformants plated in LB supplemented with X-GAL (20 µg/ml) and ampicillin. The insertion was confirmed by blue and white screening, colony PCR (see PCR conditions in **Table E3** in **Annex E**) of white colonies and sequencing. After that, the vector was extracted from an overnight grown culture using the extraction kit and transformed in *S.*

*cerevisiae* CEN.PK2-1C strain using the method described in **section 3.5**. The recombination events take place right after transformation since the CRISPR-Cas9 machinery is regulated by constitutive promoters not requiring any kind of induction.

### 3.8.3 Plasmid curing

To introduce the plasmids carrying the curcumin pathway in the knockout strain it was first necessary to remove the pCRCT plasmid. For this purpose, a positive colony (Δ*fdc1*) was grown in YPD overnight (to induce plasmid loss) and then plated in YNB supplemented with uracil and 5-FOA. When 5-FOA is added to the medium, the enzyme produced by the URA3 gene, codified by pCRCT, converts 5-FOA into a toxic compound, acting as a negative marker. Afterwards, the colonies were re-plated in the same media two more times, to ensure the plasmid loss, and finally plated in YNB +URA solid media and stored. After that, *S. cerevisiae* CEN.PK2-1C Δ*fdc1* strain was transformed and tested for curcumin production.

### 3.8.4 Knockout confirmation

In order to confirm *fdc1* knockout by CRISPR-Cas9 method, a colony PCR was performed on a plasmid cured colony (see PCR conditions in **Table E3** in **Annex E**) using specific primers to the *fdc1* gene (FDC FW and FDC REV) (**Table D2**-**Annex D**) and Phusion High Fidelity DNA polymerase. This primer pair was designed to amplify the first 761 bp of the fdc1 gene in yeast genome. The resulting fragment were purified from the agarose gel using the purification kit and sequenced with the same pair of primers. The sequencing results allowed to confirm the knockout of the *fdc1* gene.

# 4 RESULTS AND DISCUSSION

## 4.1 Evaluation of ferulic acid toxicity and conversion

Ferulic acid, like other plant phenolic compounds, is a natural antioxidant and can act as potent inhibitor against fermentative microorganisms (Borges et al., 2013). Therefore, the effect of ferulic acid in yeast growth needs to be evaluated. To produce curcumin in an engineered yeast using ferulic acid as substrate, it is first important to verify the concentration at which that compound is not toxic to the cell. It is also important to ensure that the defined ferulic acid concentration does not delay the cell growth which could reduce the efficiency of the process.

Adeboye et al. (2014) studied the inhibitory effect of ferulic acid and other phenolic compounds in an industrial strain of *S. cerevisiae*. The authors concluded that ferulic acid was too toxic at a concentration of 1.8 mM or higher. However, in a different study the same authors did not observe an inhibitory effect using the same concentration (1.8 mM) but at larger scale in a bioreactor (Adeboye et al., 2015). The researchers attributed the changes in the toxicity to the scaling up. Besides, as far as it is known, there are no other works in which ferulic acid toxicity was studied in yeast and since curcumin has never been produced in yeast there is no information about the proper ferulic acid concentrations to be used.

For this purpose, *S. cerevisiae* CEN.PK2-1C wild type strain was grown with different concentrations of ferulic acid. The fermentation was maintained during 48 h and the cell growth profile for each ferulic acid concentration can be observed in **Figure 7**.

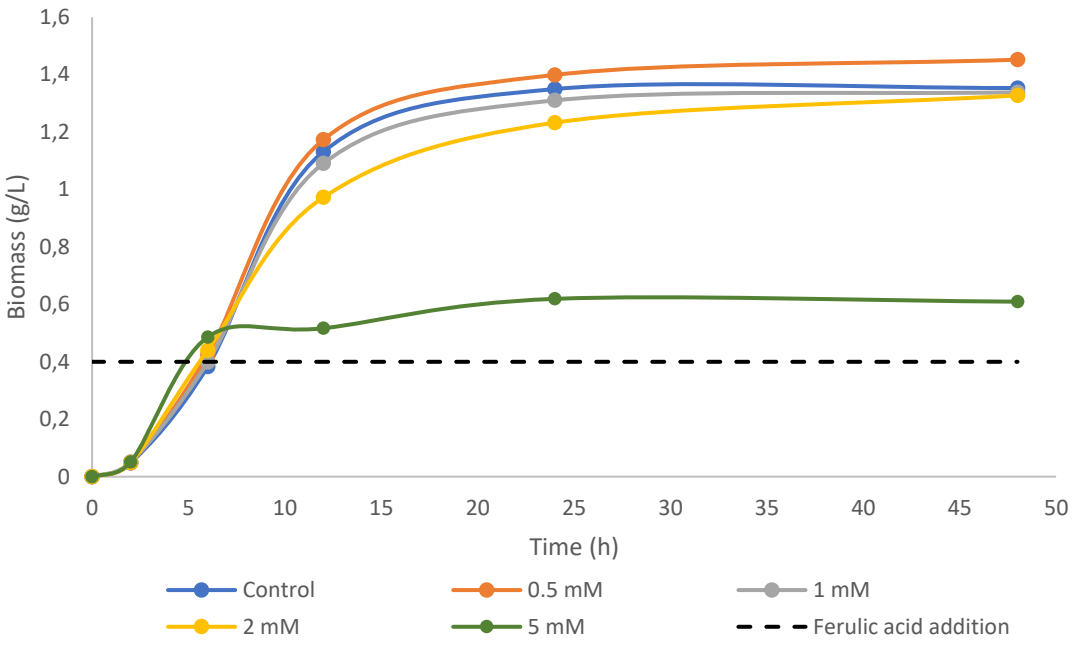



**Figure 7**: Growth curves of *S. cerevisiae* CEN.PK2-1C strain in YNB media supplemented with different concentrations of ferulic acid (0.5 mM; 1 mM; 2 mM and 5 mM). The fermentation was carried out during 48 h.

The addition of ferulic acid coincided with almost the middle of exponential phase. For the assay in which 5 mM of ferulic acid were added, growth inhibition is notorious right after addition, revealing that at this concentration ferulic acid is toxic to the yeast. The inhibition is not observed in the remaining tests since the growth curves profile resemble the control.

Between the 2 h and the 12 h (exponential growth phase) the specific growth rate of the control was 0.32 $h^{-1}$. This growth rate was similar to the rates obtained for the remaining assays: 0.5 mM (0.32 $h^{-1}$), 1 mM (0.31 $h^{-1}$) and 2 mM (0.30 $h^{-1}$). Between the addition of ferulic and the 12 h, the specific growth rates for 0.5 mM and 1 mM (0.13 $h^{-1}$ and 0.12 $h^{-1}$) resemble the control (0.12 $h^{-1}$). However, the growth rate calculated for the 2 mM assay is lower than the one of the control (0.09 $h^{-1}$). This may indicate that the addition of 2 mM of ferulic to the culture medium in some way delayed the growth of the culture. Nevertheless, after 48 h of fermentation the biomass produced in the 2 mM assay was similar to the biomass produced in the control assay. Based on these results, and taking into account a 120 h fermentation for curcumin production (ensuring the coverage of the whole fermentation process), a concentration of 2 mM of ferulic acid was selected to produce curcumin in the engineered *S. cerevisiae* strains in a first phase.

During the fermentation process samples were also taken for HPLC analysis. The goal of the sampling was to evaluate whether in fact the ferulic acid is converted to 2-Methoxy-4-vinylphenol by *S. cerevisiae*. The enzyme that may be responsible for the conversion is the FDC enzyme. The concentrations of ferulic acid during the fermentation and 2-Methoxy-4-vinylphenol at the end of the fermentation are presented in **Table 8**.

**Table 8**: Ferulic acid and 2-Methoxy-4-vinylphenol concentrations obtained during fermentation of *S. cerevisiae* CEN.PK2-1C in YNB media supplemented with different concentrations of ferulic acid. 2-Methoxy-4-vinylphenol was quantified at the end of the fermentation.

| **Assay** | **[Ferulic acid] mM** | | | **[2-Methoxy-4-vinylphenol] mM** |
|---|---|---|---|---|
| | 6 h | 24 h | 48 h | |
| Control | 0 | 0 | 0 | 0 |
| 0.5 mM | 0.451 | 0.439 | 0.409 | 0.016 |
| 1 mM | 0.798 | 0.727 | 0.659 | 0.058 |
| 2 mM | 1.674 | 1.613 | 1.538 | 0.099 |

In the experiments conducted with 0.5 mM, 1 mM and 2 mM of ferulic acid, these concentrations decrease over time and vestigial concentrations of 2-methoxy-4-vinylphenol were quantified in the end of the fermentation. In addition, 2-methoxy-4-vinylphenol concentrations increased with the ferulic concentration added to the media, as expected. Hereupon, the appearance of 2-methoxy-4-vinylphenol as well as the consumption of ferulic acid confirmed the activity of the endogenous FDC in *S. cerevisiae* CEN.PK2-1C. No 2-methoxy-4-vinylphenol was observed in the control. The concentrations of 2-methoxy-4-vinylphenol are lower when compared with ferulic the acid consumption because 2-methoxy-4-vinylphenol is converted in simpler compounds by yeast.

## 4.2 Pathways construction

In order to produce curcumin in an engineered *S. cerevisiae,* 3 biosynthetic pathways were constructed. The first two, both carrying the CUS gene from *O. sativa* together with 4CL's from different organisms. The first with 4CL isoform 1 from *A. thaliana* and the other with 4CL from *L. erythrorhizon*. The third pathway was composed by 4CL1 from *A. thaliana* and DCS and CURS from *C. longa* codon optimized for yeast. The pathways construction was carried out using traditional molecular biology techniques, PCR or molecular cloning.

### 4.2.1 CUS pathways

As mentioned before, pSP-GM1 possesses two multiple cloning sites. To construct pSP-GM1_At4CL_CUS and pSP-GM1_Le4CL_CUS, 4CL genes were cloned under control of TEF1 promoter and the respective terminator and CUS under control of PGK1 promoter, the 4CL genes were cloned first because the NotI restriction enzyme used to insert Le4CL and At4CL cuts the CUS gene. In **Figure 8** it is possible to observe the results of colony PCR used to confirm Le4CL insertion in pSP-GM1 plasmid.

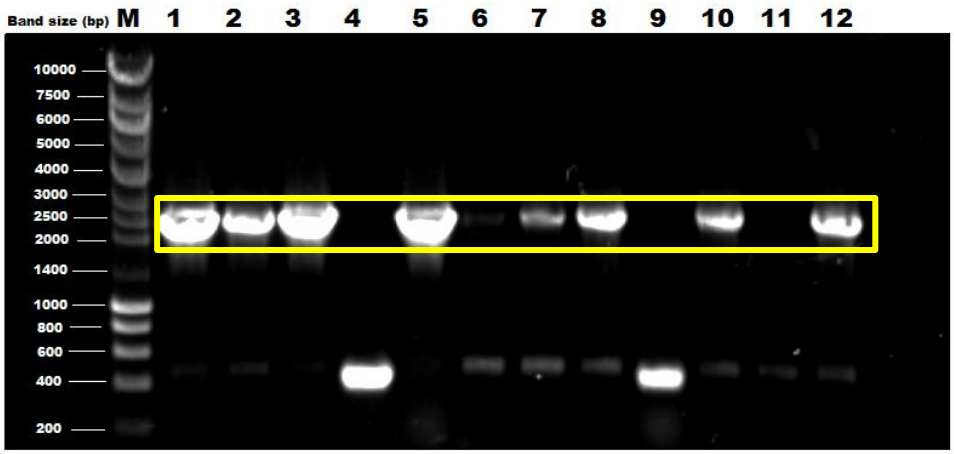



**Figure 8**: Colony PCR agarose gel 0.7 % to confirm the insertion of *L. erythrorhizon* 4CL in pSP-GM1 using TEF1p_REV and ADH1t_FW primers. M: represents the DNA ladder; 1 to 12: represents the tested colonies; Expected positive band size is 2412 bp.

The colony PCR served to prove which colonies that grew in LB selective medium possessed the plasmid pSP-GM1_Le4CL. For this purpose, an amplification of 2412 bp

(positive), Le4CL plus the amplified promoter and terminator regions, is expected. The use of two distinct restriction enzymes prevents the vector re-ligation and an amplification of 551 bp (negative) refers that these cells have been transformed with undigested vector. For pSP-GM1_Le4CL, lanes 1, 2, 3, 5, 7, 8, 10 and 12 represent positive colonies and 4 and 9 are negatives. For pSP-GM1_At4CL (data not shown) positive colonies amplification had 2161 bp. Next, positive colonies were picked arbitrarily and grown overnight in selective media. Then, the plasmid DNA was extracted and digested with one and two of the enzymes used to insert the genes in the vector. The digestion was run in agarose gel to confirm the result obtained by colony PCR. In **Figure 9** is shown, as an example, the digestion of pSP-GM1_ At4CL with NotI and PacI.

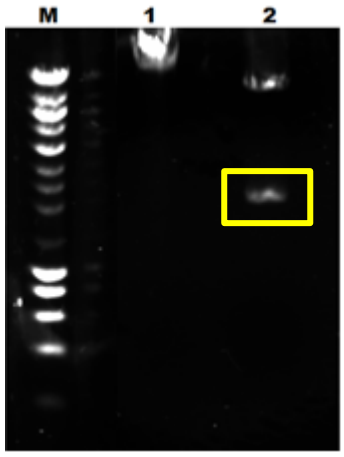



**Figure 9**: Digestion of pSP-GM1_At4CL to confirm the insertion of *A. thaliana* 4CL1 in pSP-GM1. Lanes 1 and 2 represent a the digestion of the picked positive colony; M represents the DNA ladder (NZYladder III, NZYTech). The plasmid was extracted and digested with NotI (lane 1) and with NotI and PacI (lane 2) Band sizes expected: 1- 9049 bp; 2- 7404 bp and 1718 bp.

In the lane 1, the plasmid was digested with NotI and in the lane 2 with NotI and PacI. In the well 1, a ≈10000 bp was obtained which matches the linearized pSP-GM1_At4CL size (9049 bp). In well 2 two bands are visible, one with about 7500 bp and another with ≈2000 bp which matches the size of the insertion (1718 bp). The same process was used for pSP-GM1_Le4CL and then positive plasmids were sequenced and no errors were detected in the genes sequence.

After the construction of pSP-GM1_Le4CL and pSP-GM1_At4CL, the CUS gene was inserted to complete the curcumin biosynthetic pathways. In **Figure 10** is displayed the colony

PCR agarose gel for pSP-GM1_Le4CL_CUS. In this case, the positive amplification possesses 1712 bp and the negative 491 bp.

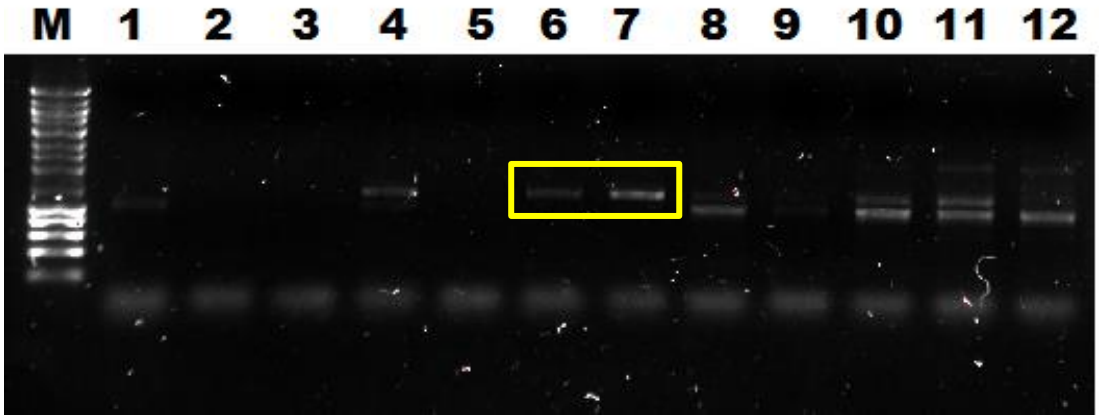



**Figure 10**: Colony PCR agarose gel 0.7 % to confirm the insertion of *O. sativa* CUS in pSP-GM1_Le4CL using PGK1p_FW and CYCt_REV primers. M: represents the DNA ladder (NZYladder III, NZYTech); 1 to 12: represents the tested colonies. Expected positive band size is 1712 bp

Lanes 4, 6, 7, 8, 9, 10, 11 clearly exhibit a band with the expected positive size, however the lanes 4, 8, 9, 10 and 11 exhibit also an unspecific amplification, perhaps in *E. coli* genome. Since colonies from lane 6 and 7 do not have unspecific amplification they were selected for the following steps. Supposedly positive pSP-GM1_Le4CL_CUS and pSP-GM1_At4CL_CUS colonies were grown overnight and the plasmids extracted. The plasmids were then digested. The digestion of pSP-GM1_At4CL_CUS is shown as an example in **Figure 11**.

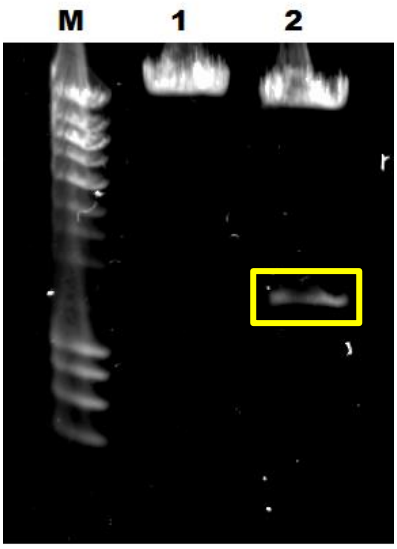



**Figure 11**: Digestion of pSP-GM1_At4CL_CUS to confirm the insertion of *O. sativa* CUS in pSP-GM1_At4CL. The plasmid of a positive colony was extracted and digested with BamHI and HindIII. Lane 1 represent the digestion with BamHI and the expect band size is 10193 bp. Lane 2 represent the digestion with BamHI and HindIII and the expected band sizes are 9049 bp and 1214 bp; M represents the DNA ladder (NZYladder III, NZYTech).

The digestion also confirmed the insertion of the CUS. In the well 1 the plasmid was digested with BamHI being obtained a band > 10000 bp corresponding to the linearized plasmid. For the digestion with BamHI and HindIII (well 2) two bands were obtained, first a ≈1400 bp corresponding to CUS (1214 bp) and the other matching the pSP-GM1_At4CL size (9049 bp). The same results were obtained for the digestion of pSP-GM1_Le4CL_CUS. The construction was also confirmed by sequencing the CUS gene. Hereupon, plasmids pSP-GM1_Le4CL_CUS and pSP-GM1_At4CL_CUS (**Figure 12**) were transformed into *S. cerevisiae* CEN.PK2-1C strain.

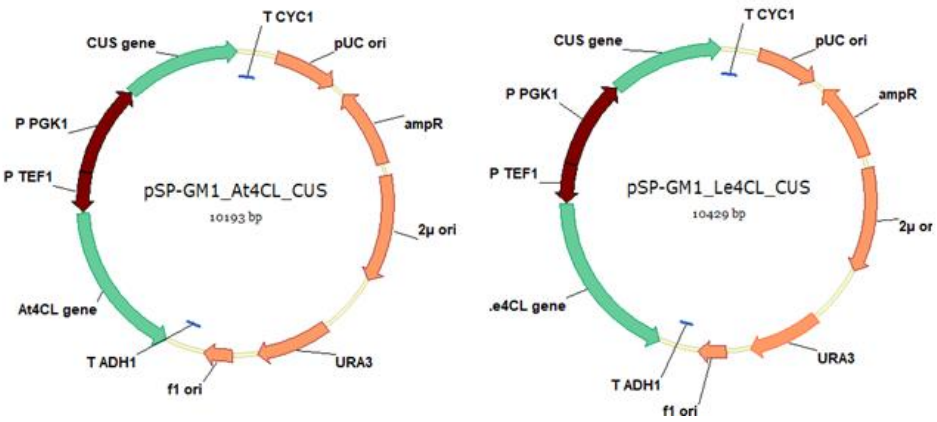


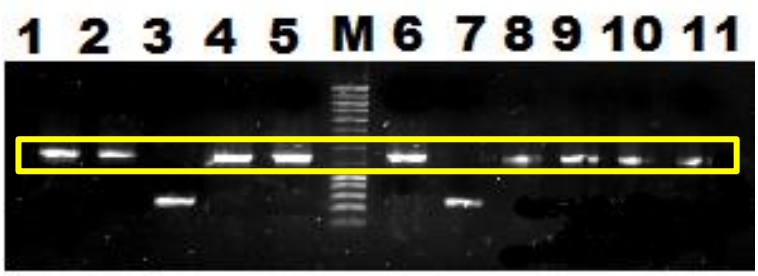

**Figure 12**: Plasmids pSP-GM1_At4CL_CUS and pSP-GM1_Le4CL_CUS

### 4.2.2 DCS and CURS pathway

To construct pSP-GM1_DCS_4CL_CURS, DCS was cloned under control of TEF1 promoter and 4CL gene was put under control of PGK1 promoter. To express the CURS gene, TDH3 promoter and synthetic terminator 27 were also inserted in the plasmid to create a third regulatory region (see strategy in **section 3.3**).

The first step in the construction of this pathway was to amplify DCS, At4CL, the promoter TDH3 and CURS1 gene. In this pathway, At4CL was inserted in a different multiple cloning site. The synthetic terminator 27 sequence was present in the primer used to amplify CURS1. The codon optimized DCS gene was first inserted in pSP-GM1 plasmid. Positive colonies were selected by colony PCR (**Figure 13, A**). Afterwards, two positive colonies were grown overnight and the extracted plasmid was digested to confirm the insertion of DCS in pSP-GM1 (**Figure 13, B**).

**[A]**




**[B]**

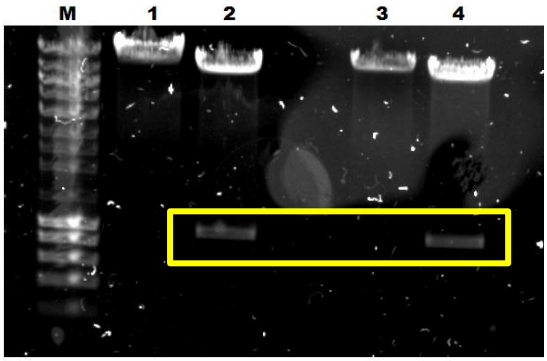
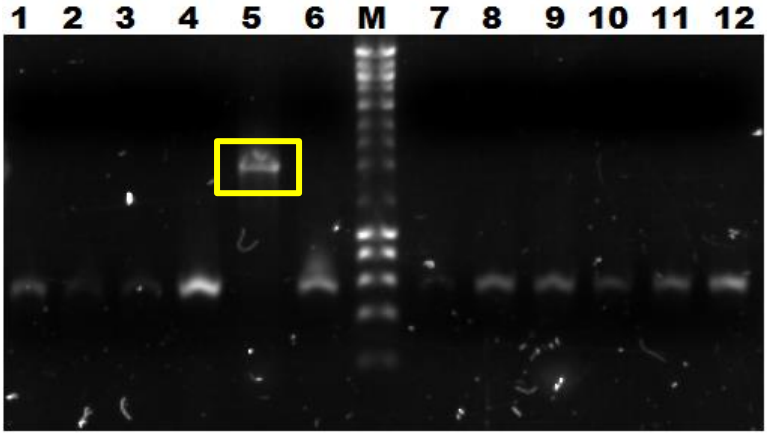



**Figure 13**: **A**: Colony PCR agarose gel 0.7 % to confirm the insertion of codon-optimized DCS in pSP-GM1 using TEF1p_REV and ADH1t_FW primers. M: represents the DNA ladder (NZYladder III, NZYTech); 1 to 11: represents the tested colonies. Expected positive band size is 1626 bp; **B**: Digestion of pSP-GM1_DCS to confirm the insertion of codon optimized DCS in pSP-GM1. The plasmids of two positive colonies were extracted and digested with NotI and SacI. Lane 1 and 3 represent the digestion with NotI and the expect band size is 8539 bp. Lane 2 represent the digestion with NotI and SacI and the expected band sizes are: 7404 bp and 1135 bp; M represents the DNA ladder (NZYladder III, NZYTech).

For this case, positive amplifications possessed 1626 bp and were obtained in all lanes except in lanes 3 and 7. The digestion confirmed the insertion of DCS in the two colonies picked, as can be visualized in the double digestion (lanes 2 and 4), as well as in linearization (lanes 1 and 3).

After sequencing, At4CL was inserted in plasmid pSP-GM1_DCS. The same process, colony PCR and digestion, was used to confirm the insertion. The positive amplification (2196 bp) was observed in **Figure 14 (A)** in the lane 5 while in the other lanes negative amplifications (499 bp) were observed. The digestion also confirmed the insertion of At4CL in pSP-GM1_DCS (**Figure 14, B**).

**[A]**




**[B]**

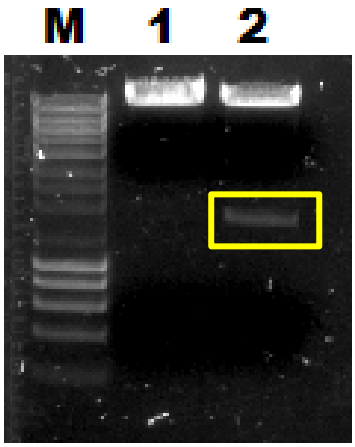
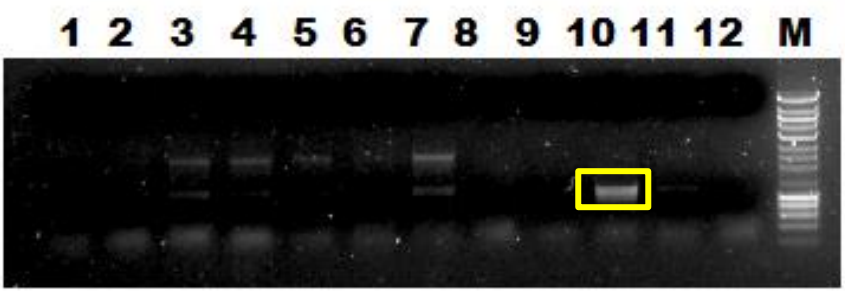



**Figure 14**: **A**: Colony PCR agarose gel 0.7 % to confirm the insertion of *A. thaliana* 4CL1 in pSP-GM1_DCS using PGK1p_FW and CYCt_REV primers. M: represents the DNA ladder (NZYLadder III, NZYTech); 1 to 12: represents the tested colonies. Expected positive band size is 2196 bp; **B**: Digestion of pSP-GM1_DCS_4CL to confirm the insertion of 4CL in pSP-GM1_DCS. The plasmid of the positive colony was extracted and digested with BamHI and XhoI. Lane 1 represent the digestion with BamHI and the expect band size is 10167 bp. Lane 2 represent the digestion with BamHI and XhoI and the expected band sizes are: 8539 bp and 1628 bp; M represents the DNA ladder (NZYLadder III, NZYTech).

After confirming by sequencing that no errors were present, the TDH3 promoter was cloned in the plasmid. The colony PCR results can be observed in **Figure 15 (A)** and the digestion in **Figure 15 (B)**.

**[A]**




**[B]**

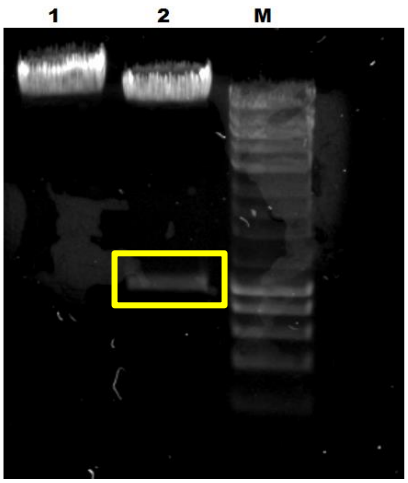



**Figure 15**: **A**: Colony PCR agarose gel 0.7 % to confirm the insertion of TDH3 promoter in pSP-GM1_DCS_4CL using pSP-GM1 FW and pSP-GM1-REV primers. M: represents the DNA ladder (NZYLadder III (NZYTech)); 1 to 12: represents the tested colonies. Expected positive band size is 1505 bp; **B**: Digestion of pSP-GM1_DCS_4CL_TDH3 to confirm the insertion of TDH3 promoter in pSP-GM1_DCS_4CL. The plasmid of a positive colony was extracted and digested with AscI and AvriI. Lane 1 represent the digestion with AscI and the expect band size is 11175 bp. Lane 2 represent the digestion with AscI and AvrII and the expected band sizes are: 10167 bp and 1008 bp; M represents the DNA ladder (NZYLadder III (NZYTech)).

Again, besides the positive amplification, in some lanes are visualized some unspecific amplification. The plasmid from colony 10 was selected for the next steps. The digestion and sequencing confirmed the insertion of TDH3 promoter making pSP-GM1_DCS_CURS_TDH3 plasmid. Afterwards, pSP-GM1_DCS_CURS_TDH3 and the amplicon composed by codon optimized CURS and the synthetic terminator 27 were also inserted. The results of the colony PCR and of the digestion of the positive colony is shown in **Figure 16**.

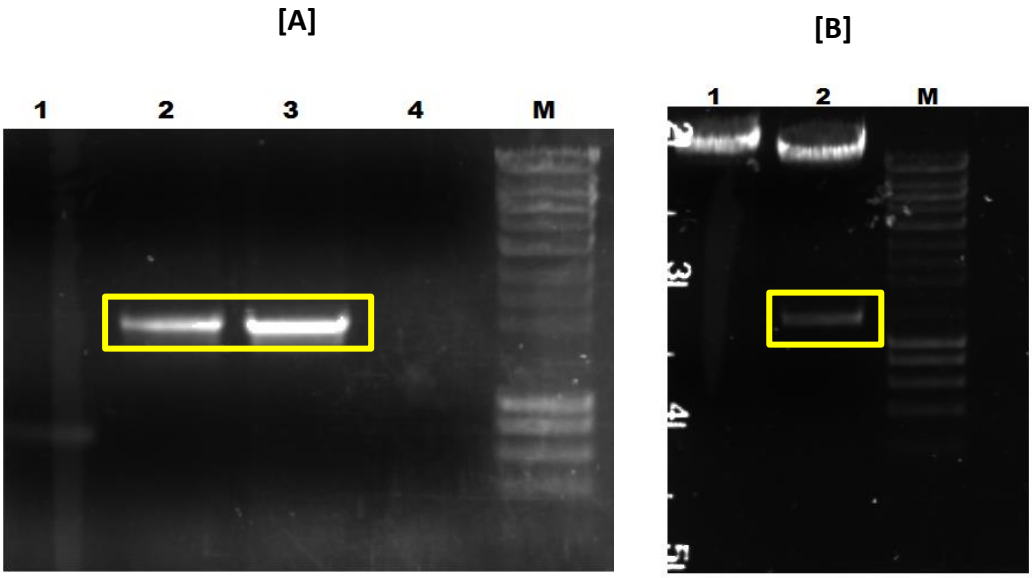



**Figure 16 : A**: Colony PCR agarose gel 0.7 % to confirm the insertion of codon optimized CURS1 with synthetic terminator in pSP-GM1_DCS_4CL_THD3 using using TDH3_middle FW and pSP-GM1 REV primers. M: represents the DNA ladder (NZYLadder III, NZYTech); 1 to 4: represents the tested colonies. Expected positive band size is 1984 bp; **B**: Digestion of pSP-GM1_DCS_4CL_CURS1 to confirm the insertion of CURS1 with synthetic terminator_27 in pSP-GM1_DCS_4CL_THD3. The plasmid of a positive colony was extracted and digested with AscI and SphI. Lane 1 represents the digestion with AscI and the expect band size is 12413 bp. Lane 2 represents the digestion with AscI and SphI and the expected band sizes are: 11175 bp and 1238 bp; M represents the DNA ladder (NZYLadder III, NZYTech).

Positive bands (1984 bp) were obtained in two lanes and a negative one (685 bp) in lane 1. The digestion of colony 3 plasmid confirmed the insertion of CURS1 with the synthetic terminator. Finally, the plasmid pSP-GM1_DCS_4CL_CURS1 (**Figure 17**) was sequenced and transformed into *S. cerevisiae* CEN.PK2-1C strain.

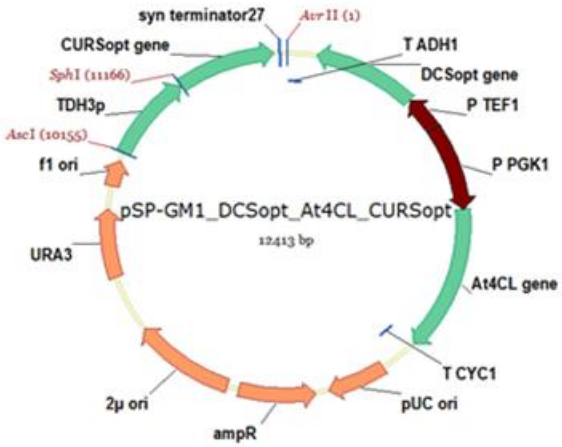



**Figure 17**: Plasmid pSP-GM1_DCS_4CL_CURS1.

## 4.3 Batch fermentation – Curcumin production

The production of curcumin in heterologous organisms has the advantage of conferring colour to the culture media due to the bright yellow colour of curcumin, which comprises an expedite way to qualitatively confirm the production of the target compound. *S. cerevisiae* does not hold a specific polyphenol transporter, thus it was expected that the produced curcumin remains inside the cells being the biomass responsible for the yellow colour of the culture. At the end of the fermentation, the yellow cultures were collected and curcumin was extracted using methanol.

### 4.3.1 Assays with 2 mM ferulic acid

In a first phase, *S. cerevisiae* CEN.PK2-1C cultures carrying the plasmids pSP-GM1_At4CL_CUS, pSP-GM1_Le4CL_CUS and pSP-GM1_DCS_4CL_CUR1S were cultivated in YNB -URA selective media and 2 mM of ferulic acid were added to culture when an $OD_{600nm}$> 0.2 was reached. The growth was followed until a continuous $OD_{600nm}$ was obtained. The growth curves of *S. cerevisiae* CEN.PK2-1C carrying the curcumin biosynthetic pathways and a control (*S. cerevisiae* CEN.PK2-1C wild strain) are shown in the **Figure 18**.

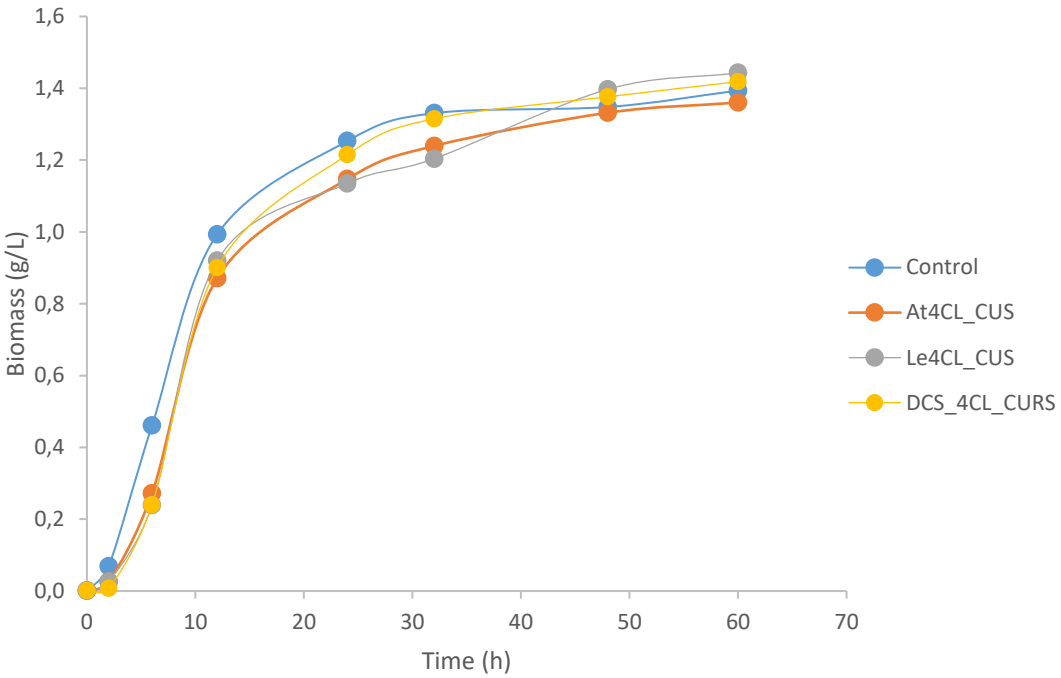



**Figure 18**: Growth curves of *S. cerevisiae* CEN.PK2-1C strain (control) and *S. cerevisiae* CEN.PK2-1C carrying the curcumin biosynthetic pathways in YNB -URA media (except for the control: YNB +URA) supplemented with 2 mM of ferulic acid.

The specific growth rates calculated for the exponential phases were: 0.093 $h^{-1}$ (Control), 0.085 $h^{-1}$ (At4CL_CUS), 0.090 $h^{-1}$ (Le4CL_CUS) and 0.089 $h^{-1}$ (DCS_4CL_CURS1). The growth specific rates of the engineered strains, as well as the maximum growth obtained were very similar to the control revealing that the plasmids did not delay the cell growth. However, regarding curcumin production, no yellow colour formation could be observed in the culture media after 120 h of fermentation. Hereupon, the concentration of ferulic acid and the formation of 2-Methoxy-4-vinylphenol in the end of the fermentation were evaluated. The results obtained are shown in **Table 9**.

**Table 9**: Ferulic acid and 2-Methoxy-4-vinylphenol concentrations obtained by HPLC after fermentation of *S. cerevisiae* CEN.PK2-1C strain (control) and S. cerevisiae CEN.PK2-1C carrying the curcumin biosynthetic pathways in YNB media supplemented with 2 mM of ferulic acid.

| | [Ferulic acid] (mM) | [2-Methoxy-4-vinylphenol] (mM) |
|---|---|---|
| **Control** | 1.667 | 0.12 |
| **At4CL_CUS** | 1.465 | 0.04 |
| **Le4CL_CUS** | 1.521 | 0.02 |
| **DCS_4CL_CURS** | 1.709 | 0.04 |

The concentration of ferulic acid obtained for the control was analogous to that obtained in the experiments with engineered strains. This showed that ferulic acid may have only been converted to 2-Methoxy-4-vinylphenol and 2-Methoxy-4-vinylphenol degradation products, thus not being used to produce curcumin. The fermentations were repeated and no curcumin was visually observed.

Typically, in fermentations performed with rich media the cell concentrations and protein expression levels are higher than in fermentations with minimal media. This fact may favour the production of heterologous compounds in engineered yeasts. Jordan and Nash (2015) studied the rate of episomal plasmid loss in yeast in the absence of selective pressure. The authors concluded that yeast retain the plasmids much longer than expected suggesting that growth in non-selective medium may be a viable process. Thus, it was decided to use rich medium to test curcumin productivity in order to identify whether higher concentrations of biomass could result in the production of curcumin. The engineered strains were inoculated into YPD (without selective pressure) and the ferulic acid was added when an $OD_{600nm} > 2$ was reached. The $OD_{600nm}$ was followed during the fermentative process resulting in the growth curves presented in **Figure 19**.

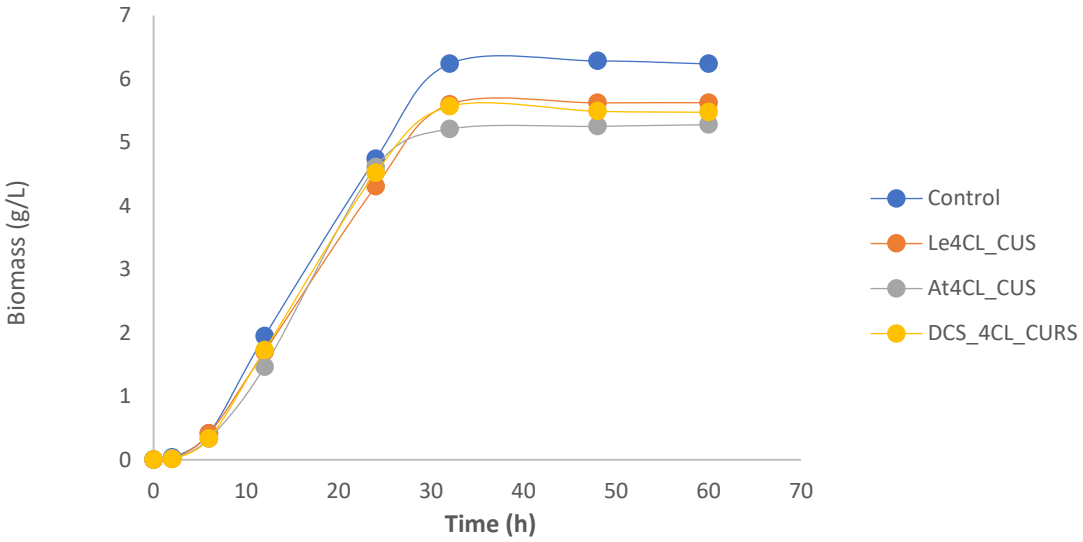



**Figure 19**: Growth curves of *S. cerevisiae* CEN.PK2-1C strain (control) and *S. cerevisiae* CEN.PK2-1C carrying the curcumin biosynthetic pathways in YPD media supplemented with 2 mM of ferulic acid. The fermentation was followed during 68 h.

As expected, the production of biomass in rich media (Control: 6.24 g/L) was higher than in minimal media (Control: 1.39 g/L). However, after 120 h of fermentation and removal of the culture media (due to the yellowish colour of YPD) no yellow colour of biomass was observed which was also confirmed by the concentrations of ferulic and 2-Methoxy-4-vinylphenol at the end of the fermentation (**Table 10**).

**Table 10**: Ferulic acid and 2-Methoxy-4-vinylphenol concentrations obtained by HPLC after fermentation of *S. cerevisiae* CEN.PK2-1C strain (control) and *S. cerevisiae* CEN.PK2-1C carrying the curcumin biosynthetic pathways in YPD media supplemented with 2 mM of ferulic acid.

| | [Ferulic acid] (mM) | [Vinylphenol] (mM) |
|---|---|---|
| **Control** | 1.570 | 0.02 |
| **At4CL_CUS** | 1.441 | 0.01 |
| **Le4CL_CUS** | 1.680 | 0.08 |
| **DCS_4CL_CURS** | 1.601 | 0.02 |

### 4.3.2 Decreasing ferulic acid concentration

Ferulic acid is a phenolic compound present in lignocellulosic biomass and is known to be toxic to cell growth and fermentative capability. Shin et al. (2011) determined the optimal substrate concentration for resveratrol production in yeast. For that purpose, the substrate, *p*-coumaric acid was added to the media in a concentration range of 8 to 80 mg/L. Using a concentration of 16 mg/L, the authors achieved the highest yield of resveratrol and superior concentrations of substrate reduced the produced resveratrol concentrations. The researchers did not identify a reason for such results but suggested that phenolic acids like coumaric or ferulic, may interrupt the central metabolism in yeast.

Hereupon, it was decided to test if with a ferulic acid concentration of 16 mg/L (0.08 mM), 25-fold lower than the one used in previous tests, the production of curcumin would be achieved. With that purpose, the recombinant strains were inoculated in YNB -URA selective media and 16 mg/L of ferulic acid were added in the beginning of the fermentation process. The yellow colour formation was visualized in the fermentation with *S. cerevisiae* CEN.PK2-1C harbouring the Le4CL_CUS pathway confirming the curcumin production (**Figure 20**). The curcumin present in the cells was extracted with methanol and analysed in HPLC (**Figure 21**).

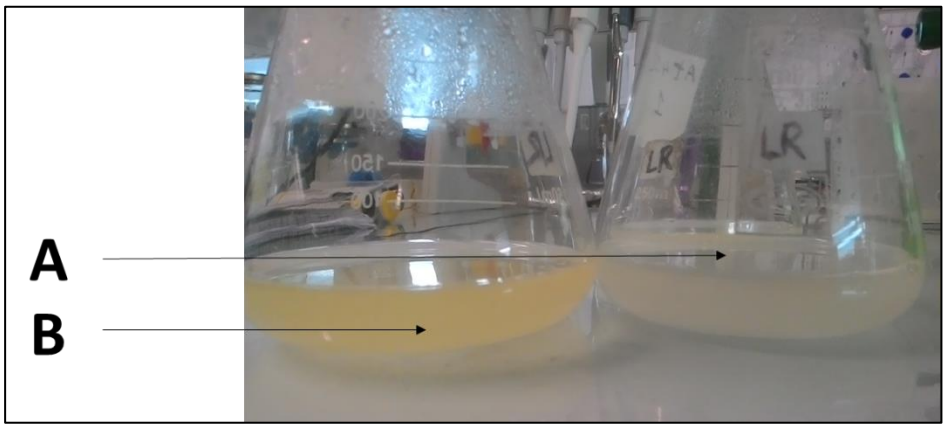



**Figure 20**: Yellow colour formation in the culture media. **A**: Control: *S. cerevisiae* CEN.PK2-1C; **B**: *S. cerevisiae* CEN.PK2-1C harbouring Le4CL_CUS pathway.

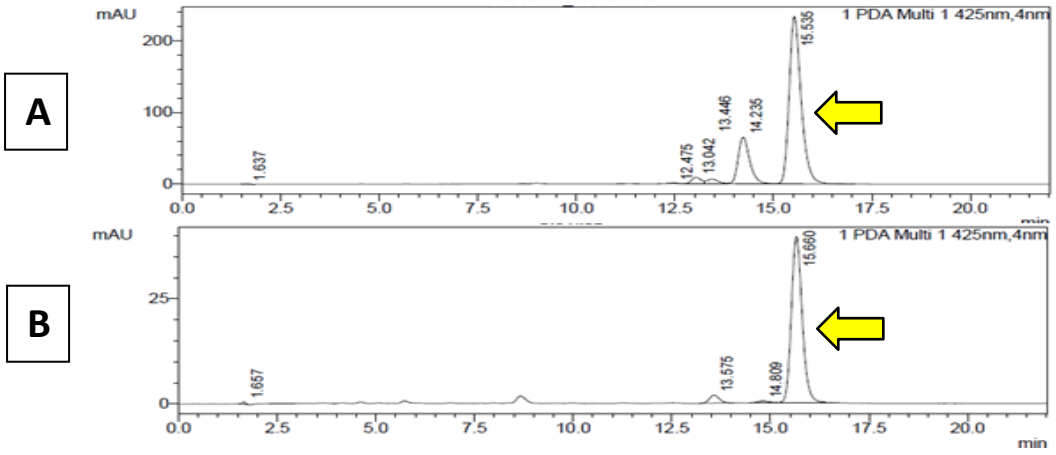



**Figure 21**: HPLC analyses of curcumin produced by recombinant *S. cerevisiae* CEN.PK2-1C carrying Le4CL_CUS pathway. **Chromatogram A** represents the authentic standard of curcumin and **chromatogram B** represents the sample that contains curcumin produced by recombinant *S. cerevisiae*. Note: The other peaks visualized in A is due to the 95 % purity of the curcumin standard.

The assays were performed in duplicate and concentrations of 87.3 ng/L and 39.7 ng/L were detected corresponding to a yield of 0.55 % and 0.25 %, respectively. The difference in curcumin productivity between the duplicates harbouring Le4CL_CUS pathway may be explained by the different plasmid copy number of the cultures, that is a common problem when episomal plasmids are used because the copy number of the plasmid will affect the level of recombinant protein production.

Afterwards, the production was also tested in a different *S. cerevisiae* strain. For that purpose, pSP-GM1_Le4CL_CUS plasmid was transformed into *S. cerevisiae* Y02321 strain. The fermentation was carried out in YNB -URA with 16 mg/L of ferulic acid. The obtained growth curve is displayed in **Figure 22**.

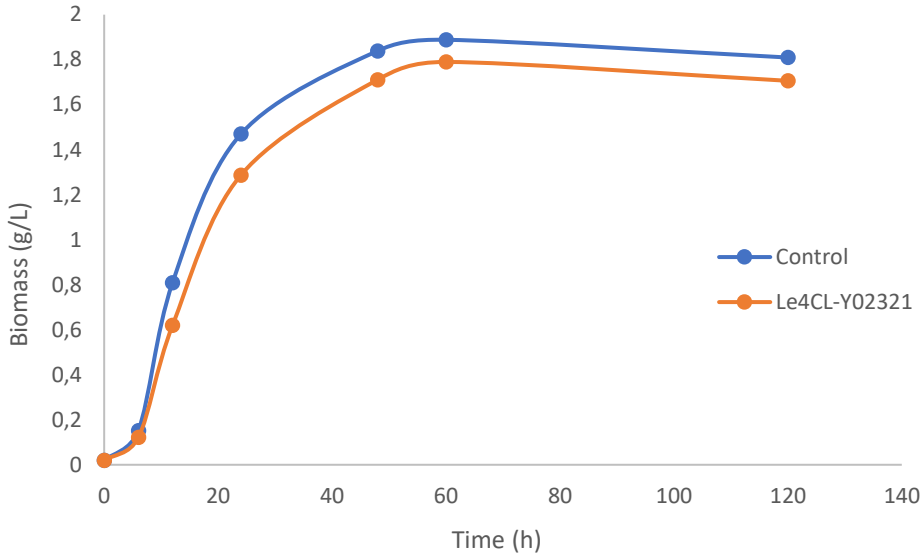



**Figure 22**: Growth curves of *S. cerevisiae* Y02321 strain (control) and *S. cerevisiae* Y02321 carrying the curcumin biosynthetic pathway Le4CL_CUS in YNB -URA (Control: YNB +URA) media supplemented with 16 mg/L (0.08 mM) of ferulic acid. The fermentation was carried out during 120 h.

The production of curcumin was also visually observed when *S. cerevisiae* Y02321 strain was used as a host. The HPLC analysis confirmed a concentration of 51 ng/L which represents a yield of 0.32 %. Comparing the growth curves of *S. cerevisiae* Y02321 strain with CEN.PK2-1C strain in YNB -URA, this strain produced a higher amount of biomass which is a desirable feature for metabolite production.

After attempts of reproducing the results in both strains using colonies from the streak plate, a yellow colour formation was no longer observed in the culture media. The fermentations were repeated under the same and with different conditions to eliminate the possibility of human error. All the culture media components were prepared fresh and different temperatures (28°C, 32°C, 35°C and 37°C) and different substrate concentrations (80 mg/L and 160 mg/L) were tested.

A reasonable explanation for this lack of reproducibility could be the plasmid copy number loss over generations. Futcher and Cox (1983, 1984) studied the maintenance and copy number of 2µm based plasmids in yeast populations and concluded that daughter cells might receive less than half of the plasmids from parent cells because yeast division is not symmetrical. The formation of cells with low copy number per cell may increase over generations. The use of colonies with high generation level may result in cultures without sufficient gene expression for curcumin production.

To prevent plasmid copy loss over generations, *S. cerevisiae* CEN.PK2-1C strain was again transformed with Le4CL_CUS pathway. After transformation, three colonies grown in the transformation plate were randomly selected and tested for curcumin productivity ensuring a minimal generational level. After fermentation, although cultures did not present a yellow colour, the methanol extraction was performed and the extract analysed by HPLC. The curcumin content quantified in each culture is displayed in **Table 11**.

**Table 11**: Curcumin produced by three different yeast cultures of *S. cerevisiae* CEN.PK2-1C carrying Le4CL_CUS pathway obtained from three colonies of the transformation plate. YNB -URA culture media was used and a concentration of 16 mg/L of ferulic was used as initial substrate concentration.

| Yeast culture | Curcumin produced (ng/L) |
|---|---|
| CEN 1 | 4.41 |
| CEN 2 | 0 |
| CEN 3 | 7.57 |

Curcumin was in fact produced in two of three yeast cultures, although at lower yields than the ones previously obtained when yellow colour was visualized. However, the fact that curcumin was detected by HPLC corroborates the successful biosynthetic pathway engineering in *S. cerevisiae*. Hereupon, a more extensive test was performed. The plasmid carrying Le4CL_CUS pathway was once again transformed into *S. cerevisiae* strains CEN.PK2-1C and Y02321 and six colonies of each strain were picked and curcumin productivity was tested using the previously conditions. The obtained results are shown in **Table 12**.

**Table 12**: Curcumin production by three different yeast cultures of *S. cerevisiae* CEN.PK2-1C and of *S. cerevisiae* Y02321 carrying Le4CL_CUS pathway obtained from six colonies of the transformation plate. YNB -URA culture media was used and a concentration of 16 mg/L of ferulic was used as initial substrate concentration. CEN1 to 6 refer to cultures using recombinant S. cerevisiae CEN.PK2-1C strain and Y1 to 6 refer to cultures using recombinant *S. cerevisiae* Y02321 strain.

| Yeast culture | Curcumin produced (ng/L) | Yield (%) |
|---|---|---|
| CEN1 | 4.01 | 0.03 |
| CEN2 | 3.02 | 0.02 |
| CEN3 to CEN6 | 0 | - |
| Y1 | 0 | - |
| Y2 | 16.7 | 0.11 |
| Y3 | 55.5 | 0.35 |
| Y4 | 5.33 | 0.03 |
| Y5 | 4.13 | 0.03 |
| Y6 | 9.09 | 0.12 |

Yellow colour formation was only observed in cultures Y2, Y3 and Y6 although curcumin was detected in a set of other samples however in low concentrations when compared with yellow cultures. Regarding the *S. cerevisiae* CEN.PK2-1C strain, the production of curcumin was only confirmed in two of the six colonies tested. On the other hand, with the strain YO2321 the production was observed in five of the six tested colonies. This result may indicate that strain Y02321 is the best producer. In fact, the use of colonies with low generation number enables the production of curcumin. The yeast culture 3 from YO2321 strain was the best producer achieving curcumin levels (55.5 ng/L and yield of 0.35 %) alike to those previously obtained.

This is the first report on the construction of a pathway to produce curcumin in *S. cerevisiae*. However, the production of curcumin visually detectable was only achieved in one of the three pathways constructed: 4CL from *L. erythrorhizon* and CUS from *O. sativa*. Nevertheless, curcumin was also detected in not yellow cultures. The fact that the yellow colour formation was not observed in At4CL_CUS and DCS_4CL_CURS cultures may indicate that curcumin is being produced but in very low concentrations. In the future, more tests with these pathways are required to confirm if in fact curcumin is being or not produced.

Although curcumin was produced using a pathway harbouring Le4CL and CUS the yields obtained were very low when compared with those obtained in *E. coli*. One possible explanation is the low amount of malonyl-CoA available to produce heterologous compounds

like curcumin. To synthesize one molecule of curcumin, two molecules of malonyl-CoA are required. In *S. cerevisiae,* malonyl-CoA concentration is maintained at low levels due to the intrinsic tight regulation of the fatty acid biosynthesis (Chen et al., 2017). The remaining malonyl-CoA may not be sufficient to produce high amounts of curcumin. The culture media used, YNB, in due to limit high biomass production. Other factors such as ferulic acid bioavailability or the extraction method, that will be discussed in 4.5, may also contributed to the low yields.

## 4.4 FDC knockout

The *fdc1* gene codifies for ferulic acid decarboxylase (FDC) in *S. cerevisiae*. This enzyme catalyses the decarboxylation of ferulic acid producing the corresponding vinyl derivate as a detoxification process. The development of a *fdc1* knockout strain may improve the curcumin productivity since the substrate consumption is prevented. CRISPR-Cas9 technology was used to cause an 8 bp deletion in the *fdc1* gene (see fdc1 sequence in **Annex D**)

The synthetized gBlock was first inserted in pCRCT plasmid using Golden Gate Assembly technique. The insertion was confirmed by blue and with screening, colony PCR (**Figure 23**) and sequencing.

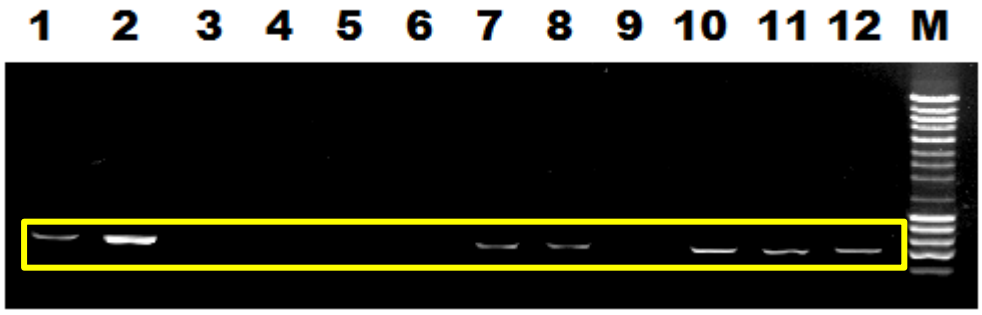



**Figure 23**: Colony PCR agarose gel 0.7 % to confirm the insertion of gBlock in pCRCT using pCRCT_FW and pCRCT_REV primers. M: represents the DNA ladder (NZYLadder III (NZYTech)); 1 to 12: represents the tested colonies; Expected positive band size is 521 bp.

Positive amplification (521 bp) was verified in more than half of the colonies tested. The sequencing results also confirmed the insertion of gBlock in pCRCT. *S. cerevisiae* CEN.PK2-

1C was transformed with pCRCT_gblock and the homologous recombination occurred during the transformation process. Afterwards, a colony was randomly chosen and a colony PCR was performed to amplify *fdc1* gene in yeast genome. The resulting band was extracted and sequenced confirming the 8 bp deletion. The alignment of the *fdc1* gene sequence and the sequencing result is shown in **Figure 24**.

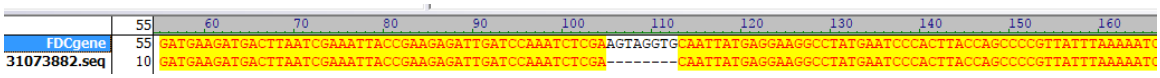



**Figure 24**: Alignment of the *fdc1* gene sequence against the sequencing results obtained by colony PCR of the fdc1 gene after pCRCT_gblock curing.

The alignment confirmed the 8 bp deletion, between bases 104 and 113 causing an alteration on the reading frame of the *fdc1* gene and an early stop in the translation. This strain was named *S. cerevisiae* CEN.PK2-1C Δ*fdc1*. The open reading frames (ORFs) of the *fdc1* gene after the deletion are displayed in **Figure 25**. As expected the 8 bp deletion, in frame 1 (same ORF as the native gene), caused an alteration on the reading frame and right after the deletion a stop codon is found. ORF2 presents the longest ORF and the protein translated is equal with the original FDC, except in the first 39 amino acids (not present in ORF2). Bhuiya et al., (2015) studied the structure of FDC enzyme. The authors identified three domains in the first 39 amino acids associated with three secondary structures of domain 1. The missing of these 39 amino acids may affect the whole enzyme structure and consequently its activity. However, as defined by Waterman et al. (1987), the nucleotide sequence composed by 6 A's, ribosome binding site, is essential in the transduction initiation. This poly(A) is not present before the starting codon in ORF2, preventing the translation of this ORF, at least with high efficiency.


**Figure 25**: 5'3' Open Reading Frames after 8 bp deletion in fdc1 gene.

Bhuiya et al. (2015) identified the FDC active site, composed by Glu285 and Arg175. The deletion of bases in one of these regions could be another suitable strategy to knockout *fdc1* gene using CRISPR-Cas9 technology.

The consumption of ferulic acid and the formation of 2-Methoxy-4-vinylphenol was studied using *S. cerevisiae* CEN.PK2-1C Δ*fdc1*. YNB supplemented with the required amino acids and uracil was used for culture media and a concentration of 2 mM of ferulic acid was added. No 2-Methoxy-4-vinylphenol formation was quantified during the fermentation and the concentration of ferulic did not decrease (data not shown). This result confirmed the knockout of the *fdc1* gene and consequently FDC inactivity.

Afterwards, the pathway Le4CL_CUS was transformed into *S. cerevisiae* CEN.PK2-1C Δ*fdc1* strain and tested for curcumin productivity. Six colonies were tested and no curcumin production was detected by HPLC using this strain. More tests are required using the knockout strain, repeat the pathway transformation for instance, or even the plasmid curing process. Due to the low production yields and to the fact that ferulic acid is not completely transformed in 2-Methoxy-4-vinylphenol by the wild strain (**Table 9**) it is not expected that the knockout, by now, has a major influence on the production.

## 4.5 Curcumin extraction

The extraction method is a crucial step in the production of heterologous compounds because an inefficient extraction may compromise the whole process. When produced in *E. coli*, curcumin was extracted using ethyl acetate. However, in yeast this method did not work since the biomass remained yellow when in contact with this solvent suggesting that the curcumin remained inside the cells perhaps because of the lack of permeability of the yeast membrane to ethyl acetate. The extraction was tested using three other different methods: in the first two the cell lysis was performed using glass beads or ethanol 70 % followed by extraction with ethyl acetate and in the third only pure methanol was used. In the **Table 13** is presented the curcumin concentration obtained from the same culture (*S. cerevisiae* Y02321 harbouring Le4CL_CUS pathway) using the three methods and the respective yield.

**Table 13**: Curcumin extracted with three different methods from the same culture (*S. cerevisiae* Y02321 harbouring Le4CL_CUS pathway, YNB -URA media supplemented with 16 mg/L of ferulic acid). The respective production yields are also presented.

| Extraction method | Curcumin (ng/L) | Yield (%) |
|---|---|---|
| Glass beads | 124.88 | 0.78 |
| Ethanol 70 % | 233.42 | 1.46 |
| Methanol | 51.01 | 0.31 |

Comparing the curcumin extracted in methods involving cell lysis (Glass beads and Ethanol 70%) with the no cell lysis method (Methanol), the differences are very significant. More than 2- fold curcumin was extracted with glass beads and more than 4-fold when ethanol lysis was performed, revealing the importance of cell disruption in curcumin extraction from yeast. Comparing the two methods with lysis, with ethanol 70 % almost 2-fold more curcumin was extracted than with glass beads. The cell lysis performed with ethanol 70 % and extraction with ethyl acetate revealed to be the most effective method to extract curcumin from yeast. The use of this method in all tests performed could have improved yields obtained in **section 4.3**.

# 5 CONCLUSIONS AND FUTURE PERSPECTIVES

## 5.1 Conclusions

The main goal of this work was to produce curcumin from ferulic acid in an engineered *S. cerevisiae*. This main objective was accomplished. A pathway composed by *L. erythrorhizon* 4CL and *O. sativa* CUS produced curcumin in two different strains: *S. cerevisiae* CEN.PK2-1C and *S. cerevisiae* Y02321. The maximum curcumin production was 233.42 µg/L. Regarding this work some other conclusions can be stated:

- Three curcumin biosynthetic pathways were successfully constructed and validated. However, two of them did not result in visible curcumin production. More studies will be needed to evaluate the production using these pathways.
- The ferulic acid toxicity was also evaluated. This compound revealed to be toxic at a 5 mM concentration. With a concentration of 2 mM the growth was only slightly delayed. In this assay, the consumption of ferulic acid and the formation of 2-methoxy-4-vinylphenol by *S. cerevisiae* CEN.PK2-1C wild type was also confirmed.
- High concentrations of ferulic acid did not contribute for curcumin production. Contrariwise, low concentrations of ferulic acid (0.08 mM) allowed the curcumin production;
- Cultures with high generation level did not contribute for the reproducibility because with the increase of the generation level the number of cells with low copy plasmid number possibly also increases;
- The efficiency of extraction methods was addressed revealing the importance of cell lysis in the curcumin extraction from yeast;
- A strain with a knockout to the *fdc1* gene was developed using CRISPR-Cas9 technology. The mutant did not consume ferulic acid and did not produce 2-methoxy-4-vinylphenol proving the FDC inactivity. This technique proved to be a simple, rapid and effective DNA editing tool;

- The mutant harbouring the Le4CL_CUS did not produce curcumin, however more tests are necessary because the mutation only prevents the consumption of ferulic acid by yeast.

In summary, besides the low yields, the construction of an engineered *S. cerevisiae* strain capable of producing curcumin from ferulic acid was successfully accomplished, as well as the development of an *fdc1* knockout strain. Overall, the results obtained in this work open a door for further work in a not yet developed field, the curcumin production in yeast.

## 5.2 Future perspectives

This work represents the first report where heterologous production of curcumin was achieved in an engineered *S. cerevisiae*. Nevertheless, the titters obtained were low, especially when compared to those obtained in engineered *E. coli.* In addition, in two of the constructed pathways there was no visible production of curcumin. More tests are required using these pathways because curcumin may not be produced in sufficient amounts to confer yellow colour to the culture but still be produced. Hereupon, the future perspectives can be framed in the optimization process both at the genetic and operational levels. Some suggestions for further research are given below:

At the genetic level:

- More efficient enzymes may increase the production yields. Therefore, different 4CL enzymes/isoenzymes should be tested. In addition, 4CL's and CUS gene sequence may be codon-optimized.
- The malonyl-CoA availability should be improved by the expression of malonyl-CoA synthase from plants or acetyl-CoA carboxylase (ACC) genes from other organisms or even the overexpression of the native ACC.
- The use of inducible promoters could also enhance the curcumin productivity, such as GAL1 or synthetic components like *Tet* promoter adapted from bacteria. This kind of systems has the advantage of controlling the enzyme expression levels and can be useful for probing the optimal expression level;

- Strategies involving plasmids with higher copy number could also improve the results. In addition, the utilization of integrative plasmids or even yeast artificial chromosomes could be tested since they provide a more stable expression platform.
- The expression of transporters may contribute to reduce the curcumin accumulation inside the cells increasing its threshold limit allowing higher production yields.
- Insert degradation tags, taken from native yeast proteins, in curcumin biosynthetic genes may increase the half-lives of the heterologous proteins which may give a boost in the enzymatic activity. Localization tags can also be inserted to localize enzymes to native organelles in yeast. Organelles are supplies of important cofactors and can sequester toxic compounds. In mitochondria, for instance, the pool of acetyl-CoA is higher than in cytoplasm which may be advantageous for the expression of ACC.
- The use of fusion proteins could increase curcumin productivity because protein::protein interactions can increase the metabolic efficiency. For example, the construction of DCS::CURS fusion protein could be tested.

At operational level:

- Other *S. cerevisiae* strains, culture media, substrate concentrations and extraction methods may be considered.
- After optimization, the next step is the scale-up where the fed-batch culture operation mode may be implemented. Low-cost substrates could also be considered to decrease the production costs and develop a more sustainable process. Agricultural wastes rich in ferulic acid like rice bran, maize bran, pineapple peels, orange peels, and pomegranate peels may be used as substrates or a biosynthetic pathway may be constructed to produce curcumin from simple substrates (e.g., glucose).
- The development of a rapid method for curcumin quantification, such as spectrophotometry would be very useful to evaluate production;
- In addition, the evaluation of curcumin localization in yeast, such as its intracellular location (e.g., using microscopy techniques).

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# 6 ANNEXES

## Annex A – Strains

**Table A14:** *Escherichia coli* and *Saccharomyces cerevisiae* strains used in this work. Genotypic information and strain source are also displayed.

| Strain | Genotype information | Source |
|---|---|---|
| *Escherichia coli* NZY5α | *fhu*A2Δ(*arg*F-*lac*Z)U169; *pho*A; *gln*V44; Φ80 Δ(*lac*Z)M15; *gyr*A96; *rec*A1; *rel*A1; *end*A1; *thi*-1; *hsd*R17 | NZYTech, Portugal |
| *Saccharomyces cerevisiae* CEN.PK2-1C | MATa; his3D1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2 | (Entian and Kotter, 2007) (Euroscarf 30000A) |
| *Saccharomyces cerevisiae* Y02321 | BY4741; MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YIL162w::kanMX4 | (Euroscarf Y02321) |
| *Saccharomyces cerevisiae* ΔFDC | MATa; his3D1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2; Δ*fdc1* | This study |

## Annex B – Plasmids

In this annex are shown all the plasmids used to construct the curcumin biosynthetic pathway as well as the plasmids used for CRISPR-Cas9 (gene knockout).

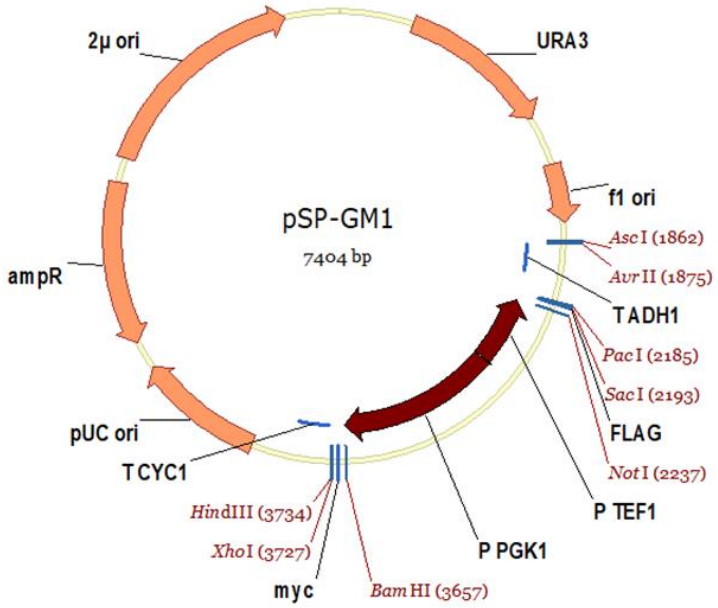


**Figure B1**: pSP-GM1 plasmid map with its main features. The restriction enzymes sites used are highlighted (red).

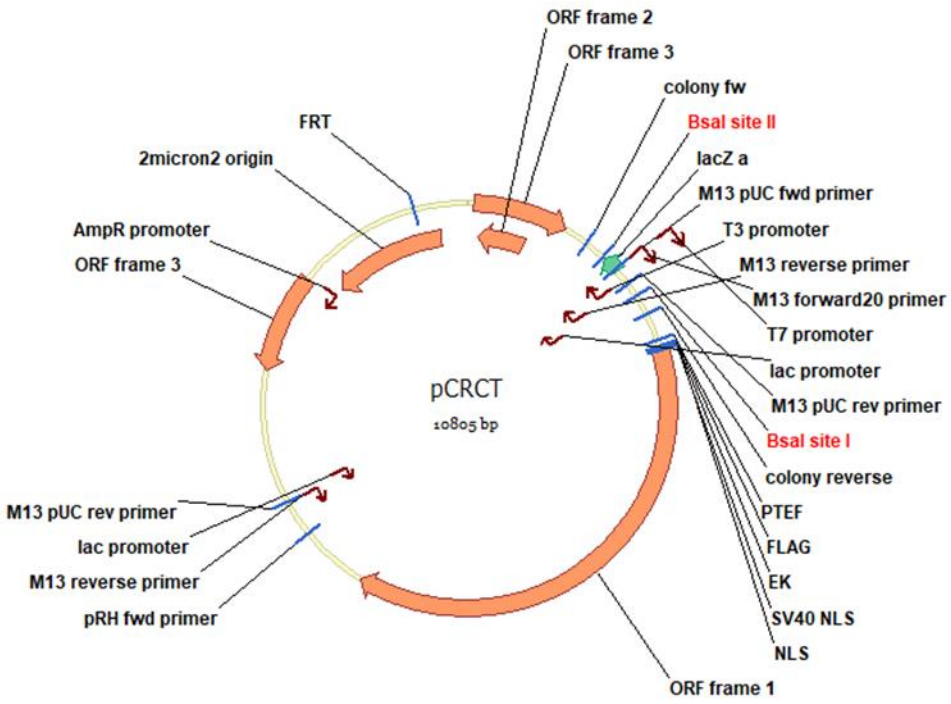


**Figure B2**: pCRCT plasmid map with its main features. The BsaI restriction sites are highlighted (red).

**Table B15**: Plasmids used in this study.

| Plasmids | Features | Source |
|---|---|---|
| pNZY29 | AmpR, rop, ori | NZYTech |
| pNZY29_DCS | pNZY29 carrying DCS codon optimized from *C. longa* | NZYTech |
| pNZY29_CURS1 | pNZY29 carrying CURS1 codon optimized from *C. longa* | NZYTech |
| pAt4CL | Plasmid carrying 4CL1 from *A. thaliana,* AmpR, ori | (Rodrigues et al., 2015) |
| pLe4CL | Plasmid carrying 4CL from *L. erythrorhizon,* AmpR*, ori* | (Rodrigues, 2014) |
| pCUS | Plasmid vector carrying CUS from *O. sativa,* AmpR*, ori* | (Rodrigues et al*.,* 2015) |
| pTDH3 | Plasmid carrying TDH3 promoter from *S. cerevisiae, AmpR, ori* | (Rodrigues, 2014) |
| pSP-GM1 | AmpR, URA3, ori, 2µ ori | (Chen et al., 2012) |
| pSP-GM1_At4CL | pSP-GM1 carrying 4CL from *A. thaliana* | This study |
| pSP-GM1_Le4CL | pSP-GM1 carrying 4CL from *L. erythrorhizon* | This study |
| pSP-GM1_At4CL_CUS | pSP-GM1_At4CL carrying CUS from *O. sativa* | This study |
| pSP-GM1_Le4CL_CUS | pSP-GM1_Le4CL carrying CUS from *O. sativa* | This study |
| pSP-GM1_DCS | pSP-GM1 carrying DCS codon optimized from *C. longa* | This study |
| pSP-GM1_DCS_At4CL | pSP-GM1_DCS carrying 4CL from *A. thaliana* | This study |
| pSP-GM1_DCS_At4CL_TDH3 | pSP-GM1_DCS_At4CL carrying TDH3 promoter from *S. cerevisiae* | This study |
| pSP-GM1_DCS_At4CL_CURS1 | pSP-GM1_DCS_At4CL_TDH3 carrying CURS1 codon optimized from *C. longa* | This study |
| pCRCT | AmpR, URA3, lacZ, ori, 2µ ori, iCas9, tracRNA | (Bao et al., 2015) |
| pCRCT_gblock | pCRCT carrying gblock | This study |

## Annex C – Codon optimized genes

The DNA sequences of codon optimized genes DCS and CURS1 are present in this annex. The genes were codon optimized for *S. cerevisiae* and synthetized by NZYTech.

**Curcumin synthase I** (CURS1) (1170 bp; GC%: 45.50)

ATGGCCAATCTACATGCGCTTAGAAGGGAACAAAGAGCTCAGGGTCCAGCAACAATTATGGCTATCGGTACTG
CTACTCCACCTAACTTATACGAACAGTCGACATTCCCGGACTTTTATTTTCGTGTTACAAACTCAGATGATAAGC
AAGAACTCAAAAAAAAGTTTAGAAGAATGTGCGAAAAGACAATGGTGAAGAAGCGTTACCTGCATCTAACAG
AGGAAATATTAAAGGAGCGTCCTAAATTATGTTCTTACAAAGAAGCCAGTTTCGACGATAGACAAGATATTGT
GGTTGAAGAAATTCCACGTTTGGCTAAGGAAGCTGCTGAAAAGGCAATTAAAGAATGGGGTCGGCCAAAATC
TGAAATTACACATCTTGTCTTCTGTTCAATAAGTGGTATTGACATGCCAGGTGCGGACTACAGGTTAGCCACTT
TGCTGGGCTTACCTCTTACAGTTAATCGTCTTATGATTTATAGTCAGGCCTGTCACATGGGTGCTGCTATGTTGC
GAATAGCAAAAGATTTGGCTGAGAACAACAGAGGCGCCCGCGTATTGGTTGTGGCGTGCGAAATCACAGTTC
TGTCTTTCAGAGGTCCAAACGAGGGAGATTTTGAAGCATTAGCTGGACAAGCGGGCTTTGGCGACGGGGCCG
GCGCGGTCGTTGTAGGTGCCGACCCGCTCGAAGGTATAGAAAAACCAATCTACGAAATCGCTGCTGCAATGCA
GGAAACAGTGGCAGAATCACAGGGTGCAGTGGGGGGCCATTTGAGGGCTTTTGGATGGACCTTTTATTTCCTT
AACCAGTTACCAGCAATTATTGCTGACAATTTGGGTAGAAGCCTTGAACGAGCACTTGCCCCACTCGGTGTCCG
CGAATGGAATGACGTTTTTTGGGTGGCCCATCCTGGTAACTGGGCAATTATTGATGCTATCGAAGCAAAGTTG
CAATTAAGTCCCGATAAATTGAGTACCGCAAGACATGTTTTCACTGAATACGGTAACATGCAGAGTGCAACCG
TTTACTTTGTAATGGATGAATTACGAAAAAGATCTGCCGTGGAGGGTAGATCAACAACTGGAGATGGTCTGCA
ATGGGGTGTTCTGTTGGGGTTTGGCCCAGGATTGTCAATTGAAACCGTGGTTCTAAGGTCCATGCCCTTGTAA

**Diketide-CoA synthase** (DCS) (1170 bp; GC%: 47.7)

ATGGAAGCTAACGGTTATAGAATCACTCACTCTGCCGATGGTCCAGCTACGATTTTAGCTATCGGCACTGCTAA
CCCTACTAATGTTGTTGATCAAAATGCTTATCCAGATTTCTATTTTAGAGTCACCAATTCAGAATACTTACAGGA
GTTAAAGGCTAAGTTTAGAAGAATCTGTGAGAAGGCCGCCATAAGAAAGCGGCATTTGTACTTGACCGAGGA
AATTCTTAGAGAAAATCCAAGTTTGCTGGCTCCAATGGCGCCCAGCTTCGATGCAAGACAAGCAATTGTGGTG
GAAGCGGTCCCCAAACTCGCTAAGGAAGCTGCCGAGAAGGCCATTAAGGAGTGGGGTAGACCTAAAAGCGA
CATCACGCATCTCGTTTTCTGCTCGGCATCTGGTATCGATATGCCAGGTTCCGATCTGCAGTTATTAAAATTGTT
GGGTCTTCCACCATCAGTCAATCGTGTCATGTTATATAACGTTGGTTGTCACGCTGGTGGTACAGCCTTGAGGG
TTGCCAAAGATTTGGCGGAAAATAATAGAGGCGCCCGTGTTCTGGCAGTTTGTAGCGAAGTTACTGTCCTTAG
TTATAGAGGTCCACACCCAGCACACATAGAATCACTTTTCGTCCAAGCCCTTTTCGGAGATGGAGCTGCCGCCT
TGGTCGTCGGCTCCGACCCCGTAGACGGAGTAGAAAGACCCATATTTGAAATCGCTTCCGCTTCACAAGTCAT
GTTACCCGAATCTGCTGAAGCCGTAGGAGGGCACTTGAGGGAAATTGGCTTGACCTTCCATTTAAAATCTCAG
TTACCATCCATTATTGCGTCCAATATCGAACAATCCTTGACTACAGCCTGCTCCCCATTGGGGCTGTCAGATTGG
AATCAATTGTTCTGGGCCGTGCACCCTGGAGGCAGGGCCATACTTGATCAAGTGGAAGCAAGATTGGGCCTTG
AAAAAGATCGTCTTGCCGCGACACGGCACGTACTGTCAGAATACGGTAACATGCAATCAGCCACAGTGTTGTT
CATTTTGGATGAAATGAGAAACAGGAGTGCCGCTGAGGGACACGCGACTACCGGCGAGGGATTGGATTGGG
GAGTGCTGTTGGGTTTTGGGCCAGGTTTGAGTATAGAAACTGTAGTCTTACATTCATGCAGGCTTAATTGA

## Annex D – The *fdc1* gene

The sequence of the *fdc1* gene is present in this annex. The sequences used in CRISPR-Cas9 technology are highlighted: The **red** sequence indicates the 8 bp deletion. The **red and underlined** sequence indicates the PAM site. Green: Guide sequence Blue: 50 bp upstream homology (the first 17 bp of the guide sequence are also included) Yellow: 50 bp downstream homology. The underlined sequences indicate the FDC primers.

**Ferulic acid decarboxylase** (FDC1) (*Saccharomyces cerevisiae*, 1512 bp, GC%: 40.3)

ATGAGGAAGCTAAATCCAGCTTTAGAATTTAGAGACTTTATCCAGGTCTTAAAAGATGAAGATGACTTAATCG
AAATTACCGAAGAGATTGATCCAAATCTCGAAGTAGGTGCAATTATGAGGAAGGCCTATGAATCCCACTTACC
AGCCCCGTTATTTAAAAATCTCAAAGGTGCTTCGAAGGATCTTTTCAGCATTTTAGGTTGCCCAGCCGGTTTGA
GAAGTAAGGAGAAAGGAGATCATGGTAGAATTGCCCATCATCTGGGGCTCGACCCAAAAACAACTATCAAGG
AAATCATAGATTATTTGCTGGAGTGTAAGGAGAAGGAACCTCTCCCCCCAATCACTGTTCCTGTGTCATCTGCA
CCTTGTAAAACACATATACTTTCTGAAGAAAAAATACATCTACAAAGCCTGCCAACACCATATCTACATGTTTCA
GACGGTGGCAAGTACTTACAAACGTACGGAATGTGGATTCTTCAAACTCCAGATAAAAAATGGACTAATTGGT
CAATTGCTAGAGGTATGGTTGTAGATGACAAGCATATCACTGGTCTGGTAATTAAACCACAACATATTAGACA
AATTGCTGACTCTTGGGCAGCAATTGGAAAAGCAAATGAAATTCCTTTCGCGTTATGTTTTGGCGTTCCCCCAG
CAGCTATTTTAGTTAGTTCCATGCCAATTCCTGAAGGTGTTTCTGAATCGGATTATGTTGGCGCAATCTTGGGT
GAGTCGGTTCCAGTAGTAAAATGTGAGACCAACGATTTAATGGTTCCTGCAACGAGTGAGATGGTATTTGAGG
GTACTTTGTCCTTAACAGATACACATCTGGAAGGCCCATTTGGTGAGATGCATGGATATGTTTTCAAAAGCCAA
GGTCATCCTTGTCCATTGTACACTGTCAAGGCTATGAGTTACAGAGACAATGCTATTCTACCTGTTTCGAACCCC
GGTCTTTGTACGGATGAGACACATACCTTGATTGGTTCACTAGTGGCTACTGAGGCCAAGGAGCTGGCTATTG
AATCTGGCTTGCCAATTCTGGATGCCTTTATGCCTTATGAGGCTCAGGCTCTTTGGCTTATCTTAAAGGTGGATT
TGAAAGGGCTGCAAGCATTGAAGACAACGCCTGAAGAATTTTGTAAGAAGGTAGGTGATATTTACTTTAGGAC
AAAAGTTGGTTTTATAGTCCATGAAATAATTTTGGTGGCAGATGATATCGACATATTTAACTTCAAAGAAGTCA
TCTGGGCCTACGTTACAAGACATACACCTGTTGCAGATCAGATGGCTTTTGATGATGTCACTTCTTTTCCTTTGG
CTCCCTTTGTTTCGCAGTCATCCAGAAGTAAGACTATGAAAGGTGGAAAGTGCGTTACTAATTGCATATTTAGA
CAGCAATATGAGCGCAGTTTTGACTACATAACTTGTAATTTTGAAAAGGGATATCCAAAAGGATTAGTTGACA
AAGTAAATGAAAATTGGAAAAGGTACGGATATAAATAA

## Annex E – PCR conditions

Information relative to the PCR conditions used to PCR-amplify the genes used in this study as well as the conditions used for colony PCR.

**Table E16**: Colony PCR conditions used to confirm the insertion of the genes in the plasmid. The polymerase used was KAPA *Taq* DNA polymerase.

| **Cycling step** | | **pSP-GM1_At4CL** | **pSP-GM1_Le4CL** | **pSP-GM1_At4CL_CUS** | **pSP-GM1_Le4CL_CUS** |
|---|---|---|---|---|---|
| Initial denaturation | | 95 °C, 10 min | | | |
| Denaturation | 30x | 95 °C, 30 s | | | |
| Annealing | | 44 °C, 30 s | 44 °C, 30 s | 49 °C, 30 s | 49 °C, 30 s |
| Extension | | 72 °C, 2 min 25 s | 72 °C, 2 min 38 s | 72 °C, 2 min 12 s | 72 °C, 2 min 12 s |
| Final extension | | 72 °C, 5 min | | | |

**Table E17**: Colony PCR conditions used to confirm the insertion of the genetic parts in the plasmid. The polymerase used was KAPA *Taq* DNA polymerase.

| **Cycling step** | | **pSP-GM1_DCS** | **pSP-GM1_DCS_At4CL** | **pSP-GM1_DCS_At4CL_TDH3** | **pSP-GM1_DCS_At4CL_CURS** |
|---|---|---|---|---|---|
| Initial denaturation | | 95 °C, 10 min | | | |
| Denaturation | 30x | 95 °C, 30 s | | | |
| Annealing | | 44 °C, 30 s | 49 °C, 30 s | 44 °C, 30 s | 45 °C, 30 s |
| Extension | | 72 °C, 2 min | 72 °C, 2 min 13 s | 72 °C, 1 min 32 s | 72 °C, 2 min |
| Final extension | | 72 °C, 5 min | | | |

**Table E18**: Colony PCR conditions used to confirm the insertion of gblock in (KAPA *Taq* DNA polymerase) and the colony PCR conditions performed in *S. cerevisiae* (Phusion High-Fidelity DNA polymerase) to confirm knockout.

| **Cycling step** | | **pCRCT_gblock** | ***fdc1* gene** |
|---|---|---|---|
| Initial denaturation | | 95 °C, 10 min | 95 °C, 10 min |
| Denaturation | 30x | 95 °C, 30 s | 95 °C, 10 s |
| Annealing | | 47 °C, 30 s | 56.3 °C, 10 s |
| Extension | | 72 °C, 2 min | 72 °C, 51 s |
| Final extension | | 72 °C, 5 min | 72 °C, 10 min |

## Annex F – Calibration curves

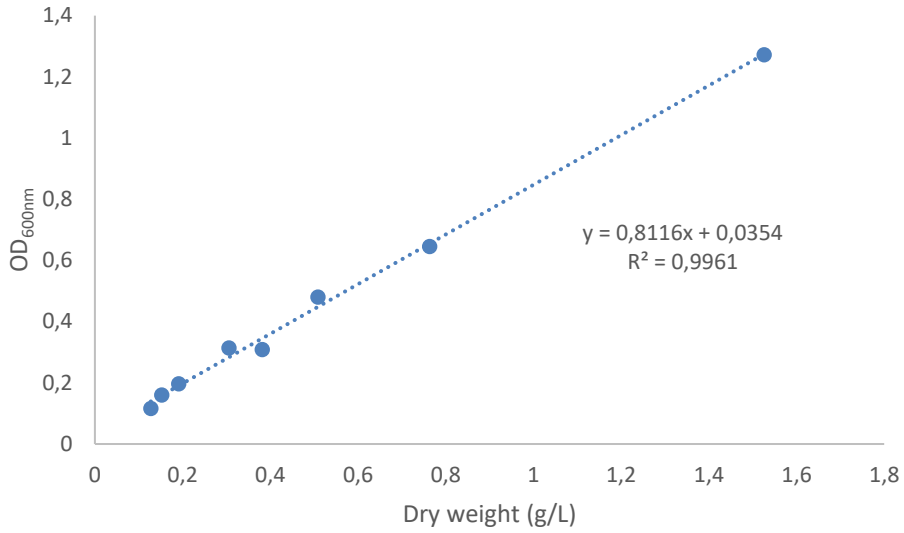


**Figure F3**: Dry weight calibration curve. The equation OD600nm = 0.8116(Dry weight) + 0.0354 was obtained with a Pearson Coefficient of Determination ($R^2$) > 0.99.

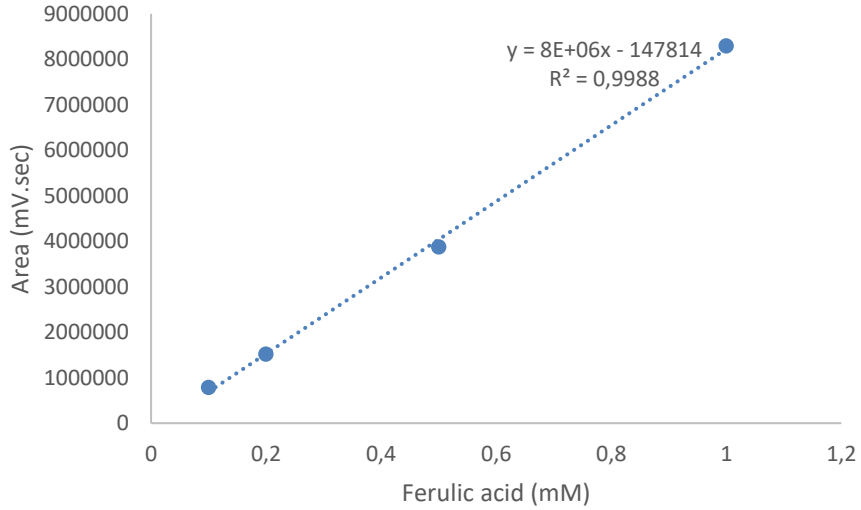


**Figure F4**: Ferulic acid calibration curve obtained by HPLC. The equation Area= 8E+06[Ferulic acid] -147814 was obtained with a Pearson Coefficient of Determination ($R^2$) > 0.99.

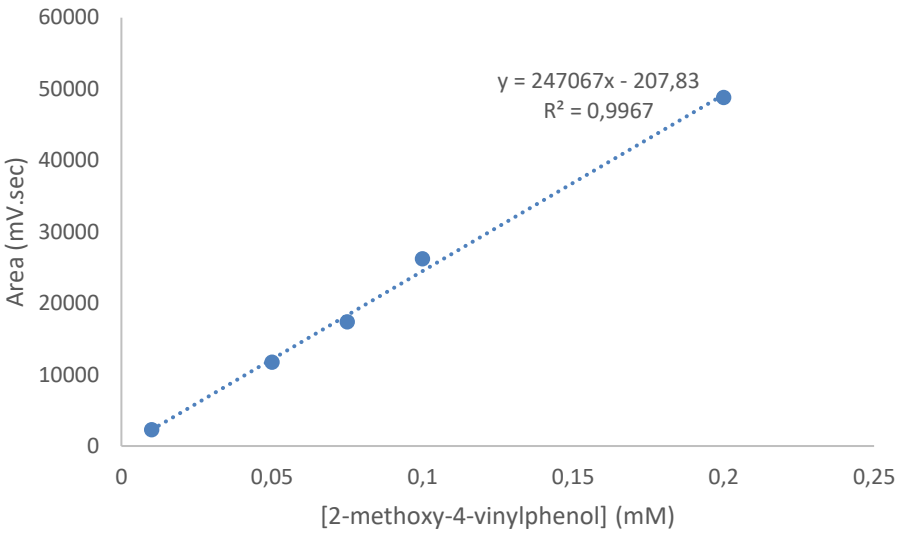


**Figure F5**: 2-Methoxy-4-vinylphenol calibration curve obtained by HPLC. The equation Area= 247067[2-Methoxy-4vinylphenol] -207.83 was obtained with a Pearson Coefficient of Determination ($R^2$) > 0.99.

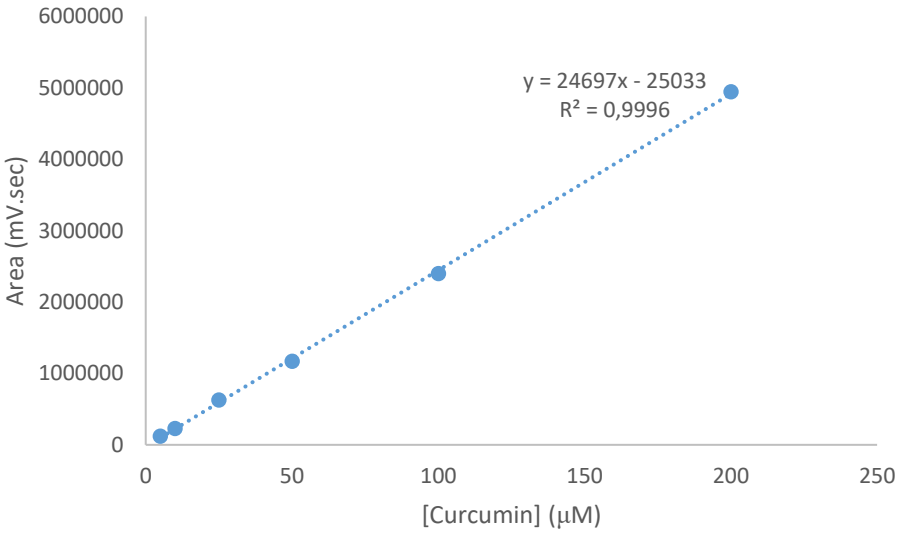


**Figure F6**: Curcumin calibration curve obtained by HPLC. The equation Area= 24697[Curcumin] -25033 was obtained with a Pearson Coefficient of Determination ($R^2$) > 0.99.