

# Optimizing the bio-production of Lycopene and $\beta$ -Carotene via metabolically engineered *Escherichia coli*

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The development in metabolic engineering and synthetic biology allows to consider new alternatives approaches to produce important compounds through living organisms. Particularly, the metabolism of *E. coli* makes possible to generate two types of carotenoids, lycopene, and  $\beta$ -Carotene. However, these compounds are not produced naturally in the bacteria, therefore the expression of some other genes that create modifications in the metabolism of *E. coli* to generate the desired compounds must be considered. Based on this, we have been able to engineer bacteria *E. coli* MG1655 to explore an optimal production of lycopene and  $\beta$ -carotene through the non-mevalonate pathway (MEP) and the over-expression of the genes *idi* and *dxs* present in this metabolic pathway by employing synthetic biology tools for the design and characterization of the best strains for the production.

Carotenoids | Production | Synthesis | Metabolism

## Introduction

Carotenoids are essential phytonutrients that are synthesized by plants, algae, fungi and some bacteria. There are approximately 1.100 types of carotenoids (1) known which can be divided into two classes, xanthophylls (contain oxygen) and carotenes (contain only hydrocarbons) (2), and they give the characteristic colors yellow, orange and red to different foods like tomatoes, carrots, pumpkins, lobster, shrimp and many more. In plants, the presence of these organic compound protect the chlorophylls of photo damage and help them to absorb light to therefore convert it into energy. In humans and some animals, some carotenoids that contain beta-ionone rings, like  $\beta$ -carotene, are involved in the absorption of vitamin A which is an important nutrient for vision, growth, cell division, reproduction and immunity.

The chemical synthesis of some carotenoids has been widely used for the production of pharmaceuticals, animal feed additives, functional cosmetics and food colorants (3). Even epidemiological approaches have been intended to prove their role in disease prevention, and their consumed is associated with a reduced risk of degenerative diseases (4) and cancer(5). Due to the important role of these compounds in the industry, scientists involved in the study of metabolic engineering have found an alternative way to produce these compounds via living organisms. Typically, this process involves the use of recombinant DNA technology to generate useful alteration of metabolic pathways to better understand and utilize the cellular pathways(6) for the production of convenient metabolites.

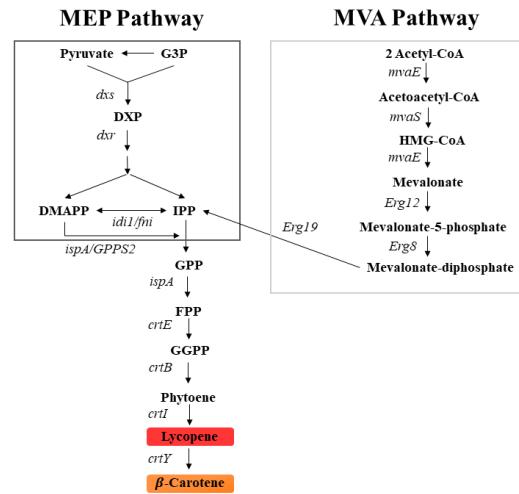


Fig. 1. Diagram of MEP and MVA pathway for the synthesis of  $\beta$ -carotene and lycopene.

In particular, the synthesis of some carotenoids, like  $\beta$ -carotene and lycopene, can be obtained by two different metabolic pathways, the mevalonate pathway (MVA) and the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP), also known as non-mevalonate pathway. A summary diagram of these two metabolic pathway is illustrated in the Figure 1. The MVA Pathway is present in eukaryotes and it is frequently used to convert Acetyl-CoA to IPP, which is subsequently isomerized to DMAPP (7). Plants have both an MVA pathway in the cytosol and a MEP pathway in the chloroplast (8). In bacteria *E. coli*, the production of isopentenyl diphosphate (IPP) and farnesyl diphosphate (FPP) is obtained in a MEP pathway. Naturally, *E. coli* does not produce either  $\beta$ -carotene or lycopene, nevertheless, the progress in the applications of metabolic engineering have allowed us to generate  $\beta$ -carotene and lycopene directly from this bacteria. Essentially, the coexpression of three genes, that can be introduced in the *E. coli* cells, named GGPP synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*), is required for the conversion of (IPP) and (FPP) to a

red-colored lycopene, and by adding an additional gene named lycopene cyclase (**crtY**), (**IPP**) and (**FPP**) can be converted to a yellow-colored  $\beta$ -carotene (7).

However, the levels of production of these carotenoids in *E. coli* are customarily low compared to the high levels produced by carotenogenic algae and microbial strains such as *Dunaliella*, *Haematococcus*, *Flavobacterium*, and *Xanthophyllomyces dendrorhous* (9). Nonetheless, some studies have shown that the over-expression of 1-deoxy-D-xylene 5-phosphate synthase (*dxs*) improves the production of lycopene (10, 11) and even a remarkable increase in the production is observed when isopentenyl diphosphate isomerase (*idi*) is over-expressed (9). For instance, the insertion of a plasmid in *E. coli* that encodes for the over-expression of *idi* and *dxs*, and another one containing the 3 or 4 genes necessary for the synthesis of lycopene or  $\beta$ -carotene, is expected to increase the level of productivity of these carotenoids.

Given that we have access to the registry of Standard Biological Parts, it is possible to characterize and utilize genetic parts that can allow us to accelerate the optimization of cellular processes for the production of a desired compound. In effect, the IGEM registry parts has approximately 20,000 documented parts like promoters, terminators, plasmid backbones, primers and many more\*. This work takes advantage of the availability of these genetic parts to engineer bacteria *E. coli* in order to produce two types of carotenoids ( $\beta$ -carotene and lycopene) via the **MEP** metabolic pathway.

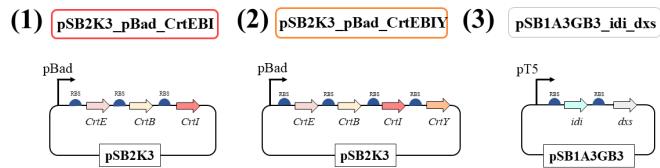
Essentially, we used three constructed plasmids which contained the needed information to provide *E. coli* with the ability of synthesize lycopene and  $\beta$ -carotene, and two additional plasmids without any gene inside to have a negative control in the experiment. We performed a double transformation with those plasmids in *E. coli* wild type MG1655 in different combinations to identify the highest level of production in the colonies by performing a drop assay. The selection of wild type MG1655 strain was based on the fact that these strains present a good metabolism and they do not contain any modification that can affect their growth. Furthermore, we analyzed each insert by colony PCR to verify the constructions, which was possible by looking into the results of the electrophoresis for different assays. Additionally, we were able to perform an extraction of the carotenoids using acetone. We repeated the same procedure for the production of carotenoids in different LB media. Finally, our results for the production of the carotenoids are supported by qualitative and quantitative measurements given that we were able to discriminate the production by observing the colors of the resulted pellets and by computing the optical absorbance for each outcome in the experiment, which allowed us then to compute the concentration of the produced carotenoids.

\* [http://parts.igem.org/Main\\_Page](http://parts.igem.org/Main_Page)

## Material and Methods

### Plasmid construction

Three plasmids, labeled pSB2K3\_pBad\_CrtEBI, pSB2K3\_pBad\_CrtEBIY and pSB1A3GB3\_idi\_dxs, were previously constructed and provided in order to explore the production of the carotenoids in *E. coli* (see Figure 2). The plasmids pSB2K3\_pBadCrtEBI and pSB2K3\_pBad\_CrtEBIY were designed by assembling the genes required by *E. coli* for the synthesis of lycopene (*crtE* + *crtB* + *crtI*) or  $\beta$ -carotene (*crtE* + *crtB* + *crtI* + *crtY*), under the control of an arabinose inducible promoter (pBad), into a backbone vector which contained an origin of replication with high copy number and a kanamycin resistance gene, and following the instructions provided from the Registry of Standard Biological Parts Distribution Kits†. The plasmid pSB1A3GB3\_idi\_dxs was built by assembling the *idi* and *dxs* genes, under the control of a IPTG inducible promoter (pT5), using the standard Golden Gate one-pot reaction protocol with the type IIS restriction enzyme BsaI and the T4 DNA ligase into a backbone vector which contained an origin of replication with high copy number and an Ampicillin resistance. Furthermore, two additional vectors, pSB2K3\_pBad (Kanamycin gene) and pSB1A2\_pLac (Ampicillin gene), were designed with a pLac and pBad promoter respectively to be used as a negative control because they produce a null expression.



**Fig. 2.** Illustration of the main parts used for the construction of the plasmids inserted in *E. coli* for the synthesis of Lycopene and  $\beta$ -Carotene.

### Analysis by PCR and electrophoresis

Firstly, a DNA purification of the plasmids pSB2K3\_pBad\_CrtEBI, pSB2K3\_pBad\_CrtEBIY, pSB1A3GB3\_idi\_dxs, pSB2K3\_pBad and pSB1A2\_pLac was made by extracting them from cloned bacteria *E. coli* MG1655 that contained the plasmids. Then, in a 1.5 ml tube was mixed 500ng of purified plasmid DNA, 1  $\mu$ l of enzymes FastDigest, 2  $\mu$ l FastDigest Green Buffer 10 $\times$ , 20 $\mu$ l H<sub>2</sub>O and later on incubated at 37° for 30min. This digestion mixture was loaded on a 1% agarose gel and analyzed by electrophoresis to check the constructions. Finally, PCR of the purified plasmids with the forward and reverse primers illustrated in the table 1 was carried on by aliquoting 20  $\mu$ l of PCR mix (8  $\mu$ l H<sub>2</sub>O, 10  $\mu$  DreamTaq

† <http://parts.igem.org/>

Green PCR Master Mix (2X), 1 pmol/ $\mu$ l Forward Primer, 1 pmol/ $\mu$ l Reverse Primer) into each PCR tube. Then 1  $\mu$ l of the plasmid DNA was added to perform the PCR reaction using a thermocycler with a selected PCR program. When this program was finished, we loaded 10  $\mu$ l on a 1% agarose gel and a voltage differential was applied in order to be analyzed by electrophoresis.

**Table 1. Primers**

Forward	Reverse
VF2: tgccacctgacgtctaagaa	VR: attaccgccttgagtggc
PbadDir: atactccgcattcagaga	CrtErev: ctgaaccaatccatgc
CrtIdir: cggatgttacccgtttg	CrtYrev: gacccgtggcatccatgataa
idi-SeqDir: ttcaagtccgttgtatgtatg	dxs-SeqRev: cgcgacaatgggttgttgc

## Media

**LB Luria/miller** (per liter: 10g/l Tryptone, 5g/l Yeast extract, 10g/l NaCl, 7,0±0,2 pH) was used for the transformation of the cells, in the LB-Agar plates for Drop Assay and for the seed culture of the production of the carotenoids. The following six media along with the **LB Luria/Miller**, were used to cultivate the transformed bacteria to test the best media for the optimal production of the carotenoids: **LB Lennox** (per liter: 10g/l Tryptone, 5g/l Yeast extract, 5 g/l NaCl, 7,0±0,2 pH), **Terrific-Broth-media (TS)** (per liter: 12g/l Tryptone, 24g/l Yeast extract, 4g/l Glycerol, 7,2±0,2 pH, 12.54 g/l K<sub>2</sub>HPO<sub>4</sub>, 2.31 g/l KH<sub>2</sub>PO<sub>4</sub>), **Tryptone-Soya-Broth (TS)** (per liter: 5g/l Peptic digesto of soybean meal, 5 g/l NaCl), **Tryptone Yeast Extract (TY)** (per liter: 10g/l Tryptone, 5g/l Yeast extract, 0,5 g/l NaCl, 7,1 pH), **Mix1** (per 400ml: 80ml M9 salt solution (5X), 4 g/l Glucose, 0,1 mM CaCl<sub>2</sub>, 2mM MgCl<sub>2</sub>) and **Mix2** (per 400ml: 80ml M9 salt solution (5X), 0,4%(v/v) Glycerol, 0,1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>). Additional ampicillin and kanamycin antibiotics were added at the following concentrations 100  $\mu$ g/ml and 25  $\mu$ g/ml respectively together with different concentrations of the inducers, specifically arabinose at 0 M, 1,5 mM or 15 mM and IPTG at 0 M, 10  $\mu$ M, 100  $\mu$ M or 1 mM.

## Growth conditions and transformation of competent cells

We inoculated 3ml of growth media to the wild type *E. coli* cells MG1655 to be growth in a shaking incubator at 37° C. Later on, 0.5 ml of the pre-cultured *E. coli* was put in 50 ml sterile LB broth. The growth in the cells was determined by measuring the optical density with a wavelength of 600 nm (OD<sub>600nm</sub>) to stop the growth once the OD600 indicated values between 0.3 and 0.4 to ensure that the cells were kept in the exponential

phase. Each sample was diluted in LB (Luria/Mille) with a ratio 1:10 to measure the OD<sub>600nm</sub>. Subsequently, the *E. coli* cells were centrifuged at 5000g in a pre-cooled rotor and then re-suspended in 1ml of ice-cold Buffer CCMB80 to later be saved at -80° C. We proceed with the transformation by adding 3 $\mu$ l of a purified plasmid (previously prepared through the extraction from the *E. coli* MG1655 bacteria that contained the plasmid) into 100 $\mu$ l of the chemical competent *E. coli* cells for each transformation. They were incubated on ice for 20min to then be placed on a heat surface for 30 seconds at 42°C to create a heat-shock in the cell. Afterwards, 500  $\mu$ l of LB Media (Luria/Miller) was added to each transformation and were incubated for 1 hour at 37°C in a shaking incubator to make them growth.

## Carotenoids extraction with acetone

15ml of bacterial cultured was centrifuged for 15min at 4000g. The resulting supernatant was removed with a micro-pipette and the bacteria were centrifuged 5min more at 4000g. 0.3 g of glass bead (1mm diameter) and 2ml of acetone were added. The assay was vortex for 5min and centrifuged at 4000 rpm for 5min. For the first extraction, the supernatant was removed and to the pellet was added 2ml acetone and repeated the vortex and centrifuge mentioned previously. For the second extraction, the supernatant was removed and combined along with the one of the first extraction to be centrifuged at 14000 rpm for 1min. The first and the second extraction were combined and then we measured the absorption for each outcome using a spectrophotometer (each sample was diluted in LB (Luria/Miller) media with a ratio 350:1500). Later on, we did the same procedure by using 7 different LB media available in the room (each sample in this case was diluted in LB media (Luria/Miller) with a ratio 1:10, in order to measure their respective absorbances).

## Analytical methods

Using a spectrophotometer, we measured the absorption as a function of the wavelength, and the OD<sub>600nm</sub> of each sample appropriately diluted to determine the production of the carotenoids in each of the media used for each sample. The data obtained from the absorption was normalized by the OD<sub>600nm</sub> of each result and multiplied by the respective dilution.

## Results & discussion

### Plasmid quality control

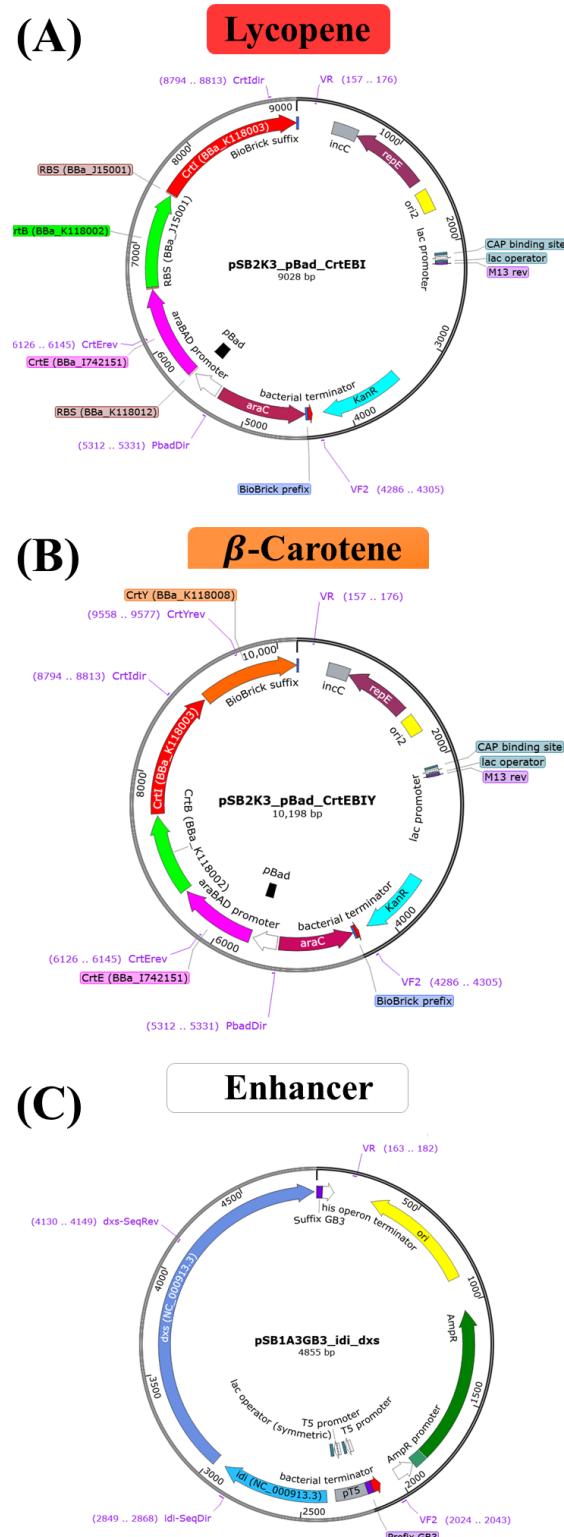
In order to perform a sequence analysis, the vectors used in the transformations, labeled as pSB2K3\_pBad\_CrtEBI, pSB2K3\_pBad\_CrtEBIY,

pSB1A3GB3\_idi\_dxs, pSB2K3\_pBad and pSB1A2\_pLac, were computationally reconstructed as is shown in figure 3 and 4. These plasmids contained several characteristics that are important to remark. First of all, the introduction of antibiotics allowed us to select only the colonies that were successfully transformed, which is essential for the results of the following sections. Second, the plasmids pSB2K3\_pBad\_CrtEBI and pSB2K3\_pBad\_CrtEBIY encoded for the production of lycopene and  $\beta$ -carotene respectively, and they contained a pBad promoter which is induced by the presence of arabinose in the media. Third, the presence of the plasmid pSB1A3GB3\_idi\_dxs, along with some of the plasmids pSB2K3\_pBad\_CrtEBI or pSB2K3\_pBad\_CrtEBIY, is expected to increase the production of the carotenoids due to the overexpression of the genes *idi* and *dxs*. Finally, the fourth feature worth noting is that a double transformation of one of these last 3 mentioned plasmids with pSB2K3\_pBad or pSB1A2\_pLac must coexist inside the cell if the plasmids have a compatible origin of replication, otherwise it may happens that the replication machinery of the cell could be splitted, therefore decreasing the expression of the genes of interest (12).

Once the sequences of the computational reconstructed plasmids were analyzed, we performed a PCR analysis to verify the correct design for each the plasmids by using the primers displayed in the Table 1. These primers were detailed in the constructed plasmids shown in figures 3 and 4 where was possible to measure the corresponding distances in base pairs between them. The point in this part was to confirm that the plasmids contained the right control sequences for bacteria to express the genes it contains when the inducers are added into the media. The results are displayed in the lower part of figure 5. As we can observe, from the electrophoresis results of figure 5, we were able to obtain the expected size for each combination of primers (labeled C1 R1, C2 R1, C3 R2, C4 R2, C3 R3, C4 R3, C5 R3, C1 R4, C6 R5) and the length for each of the plasmids (labeled R1, R2, R3, R4, R5). For instance, once the construction for each of the plasmids used was verified, we proceeded with the drop assay analysis.

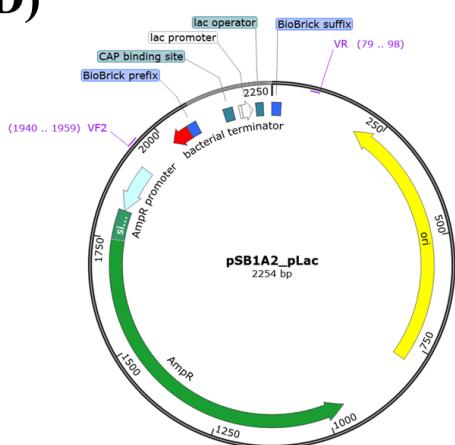
### Drop assay

When the *E. coli* MG1655 strains were double transformed with different combinations of the plasmids pSB2K3\_pBad\_CrtEBI, pSB1A3GB3\_idi\_dxs, pSB2K3\_pBad, pSB2K3\_pBad\_CrtEBIY and pSB1A2\_pLac displayed in the table of the first figure in 6, we proceeded to do a drop assay in LB plates which contained kanamycin, ampicillin and different concentrations of arabinose and IPTG. Afterwards, we measured qualitatively the produced carotenoids by observing at the colors obtained in the plates. These

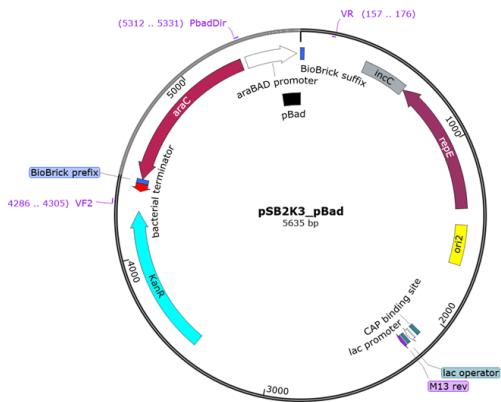


**Fig. 3.** Construction of the parts used for the synthesis of Lycopene and  $\beta$ -Carotene. **(A)** Design of the vector used for the synthesis of Lycopene, where the genes *crtE*, *crtB* and *crtl* where inserted in a empty plasmid pSB2K3 that encodes for kanamycin resistance. **(B)** Design of the plasmid used for the synthesis of  $\beta$ -Carotene, similar to the plasmid used for the synthesis of lycopene but with an additional gene *crtY*. **(C)** Constructed vector for the over-expression of the genes *idi* and *dxs*, which is expected to increase the production of either Lycopene or  $\beta$ -Carotene.

(D)



(F)



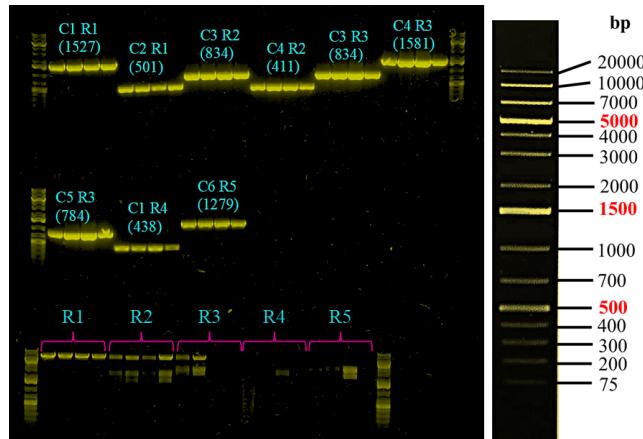
**Fig. 4.** Plasmids used for the negative control. **(D)** Constructed vector containing an IPTG inducible promoter pLac inserted in backbone plasmid pSB1A2 which contains an ampicillin resistance gene. **(F)** Constructed vector containing an inducible arabinose promoter pBad in backbone plasmid pSB2K3 which contains an kanamycin resistance gene.

observations lead to the following reasoning:

- In absence of IPTG and arabinose in the media, we observed a light orange color in the drop for the combination **a+e**, and this orange becomes a little darker with **b+e**. These results suggest that there is some basal level expression of the genes necessary for  $\beta$ -carotene production. For instance, the promoters pt5 and pBad are leaky because they are not fully repressing the expression of the genes contained in the plasmids pSB2K3\_pBad\_CrtEBIY and pSB1A3GB3\_pT5\_idi\_dxs when their inducers are not presented. Besides, we were not able to distinguish the color for the combinations **a+d** and **b+d**.

- In absence of IPTG and low concentration of arabinose (1.5mM), the drop for the combination **a+d** seems to be pink and it becomes a darker pink in the drop of

	C1	C2	C3	C4	C5	C6
Primers: F R	VF2 VR	pBadDir VR	pBadDir CrtERev	CrtDir VR	CrtDir CrtYRev	idi-SeqDir dxs-SeqRev
R1	pSB2K3 pBad	<b>1527 bp</b>	<b>501 bp</b>	-	-	-
R2	pSB2K3 pBad_CrtEBI	-	-	<b>834 bp</b>	<b>411 bp</b>	-
R3	pSB2K3 pBad_CrtEBIY	-	-	<b>834 bp</b>	<b>1581 bp</b>	<b>784 bp</b>
R4	pSB1A2 pLac	<b>438 bp</b>	-	-	-	-
R5	pSB1A3GB3 pT5_idi_dxs	-	-	-	-	<b>1279 bp</b>



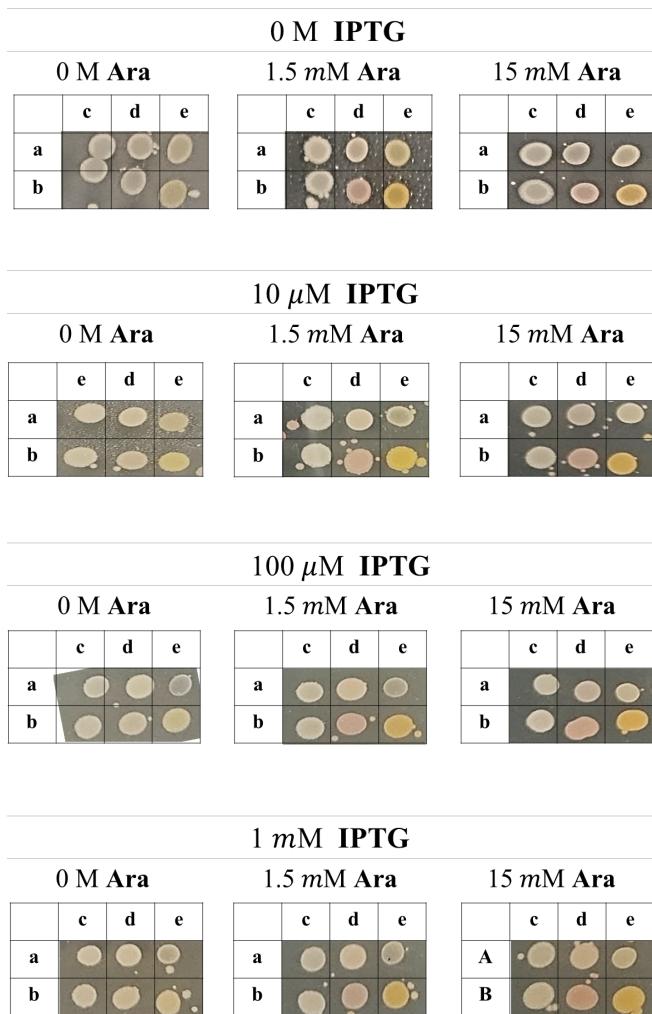
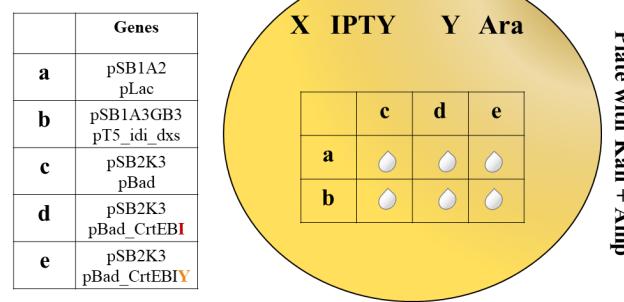
**Fig. 5.** Results of the PCR analysis to check the construction of the plasmids used for the production of the carotenoids. **Up:** Identification of the length between the forward (F) and reverse (R) primers for each plasmid. **Down:** Gel electrophoresis results of restriction digestion where it was verified the corresponding size in bp between the primers considered in table 1 and the designed plasmids displayed in figure 3 and 4.

**b+d.** These combinations encoded the genes necessary for the production of lycopene, which are controlled by the arabinose inducible promoter pBad, and the bacteria that produce lycopene is expected to color red. The darker color observed in **b+d** is obtained because of the leakiness in the promoter pT5 that allows to express the genes *idi* and *dxs* which are expected to increase the production of the carotenoids. Furthermore, we observed that the drop for the combination **b+e** colored orange, as a result of the expression of the genes in **e** for the synthesis of  $\beta$ -carotene. Moreover, the bacteria in the drop for the combination **a+e** presented an orange less intense than in the drop **b+d** which shows how important are the genes *idi* and *dxs* for the enhancement of the carotenoids production.

- When the concentration of arabinose was increased to 15mM in absence of IPTG, the color in the drops for the combinations **b+d** and **b+e** showed to be darker for either lycopene (red) or  $\beta$ -carotene (orange). This fact represents an expected result given that once the concentration of the inducers in the media is increase (not toxic for cells), the negative promoters are turning ON and the production of the recombinant proteins increase.

**X** = 0 M, 10  $\mu$ M, 100  $\mu$ M, 1 mM

**Y** = 0 M, 1.5 mM, 15 mM



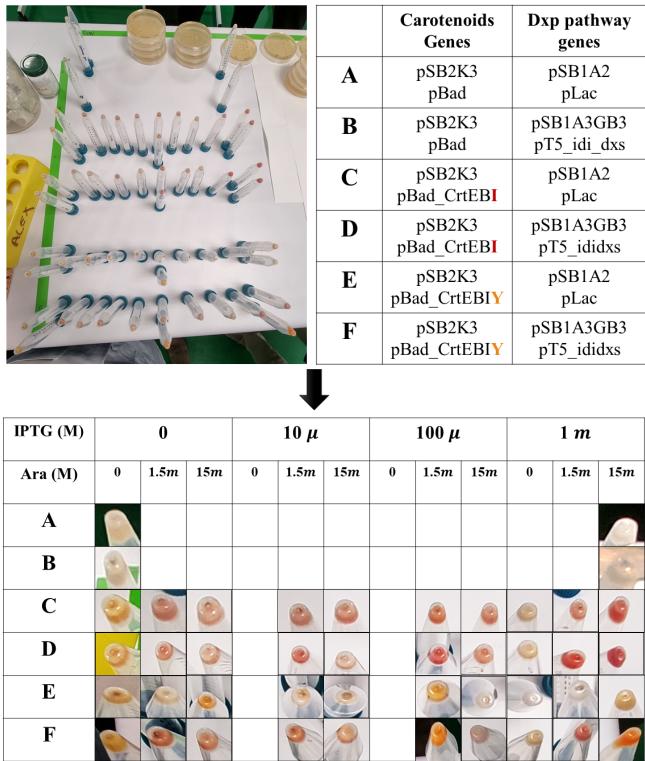
**Fig. 6.** Visual analysis of the double transformed bacteria *E. coli* with the plasmids **a**(pSB1A2\_pLac), **b** (psB1A3GB3\_pT5\_idi\_dxs), **c** (pSB2K3\_pBad), **d** (pSB2K3\_pBad\_CrtEBI) and **e** (pSB2K3\_pBad\_CrtEBIY) at different concentrations of IPTG and arabinose. Each 3x6 table represent the result obtained by cultivating the bacteria in a LB plate containing kanamycin and ampicillin. Additionally, each drop represents a colony of bacteria containing a combination of two of the plasmids **a**, **b**, **c**, **d**, **e** and **f**.

- Despite that we were expecting to observe a darker orange or red color as we increased the concentration of IPTG, we did not see any difference in the colors. For instance, the observations in the drop assay are not enough to conclude an increase in the production of the carotenoids when the concentration of IPTG was increased. On account of this, we require additional techniques that can allow us to quantify the production. One of the most common is spectrophotometry, which is based on the measurement of the absorption of light of a given sample to determine its concentration and respective color. This technique is used in the following sections.

### Carotenoids production in LB media

The results of the extraction of the carotenoids from the engineered bacteria *E. coli* MG1655, cultivated in LB media (Luria/Miller) along with different concentrations of IPTG and arabinose is displayed in Figure 7. Six double transformations with different plasmids (label as **A**, **B**, **C**, **D**, **E**, **F**) were performed , see Figure 7 Up right. The table in the Figure 7 Down is a zoom in the results for each outcome displayed in Figure 7 Up left. We could observed that no color was appreciated either in absence or present of IPTG or arabinose for the results of the cells transformed with the plasmids in **A**, because the information contained in those plasmids lacks of any desired gene for the production of either lycopene or  $\beta$ -carotene. A similar result was obtained by considering the cells transformed with the plasmids in **B**. Despite that one of the plasmids contained in **B** is encoded the information for the *idi* and *dxs* genes present in the MEP pathway (Figure 1), the absence of the genes necessary for the synthesis of the carotenoids (*crtE*, *crtI*, *crtI*, *crtY*) makes not possible for bacteria to generate these compounds naturally. For instance, the plasmids in **A** and **B** represented a negative control in our experiment.

Eventually, we visualize a notable pink-reddish color for the products of lycopene by *E. coli* MG1655 transformed with the plasmids in **C** and **D**. A curious result obtained was a light orange color in the tubes when there was not presence of arabinose and IPTG. In this case, we were expecting to observe or not color or a light pink color due to the leakiness of the promoter pBad present in the plasmids contained in **C** and **D**, as it was observed in the drop assay analysis. For instance, we concluded that this result could be due to a wrong manipulation while we were working with the plasmids (Insertion of the plasmid encoding for  $\beta$ -carotene), and the incorporation of the inducer to the media. In contrast, when arabinose was added, with a concentration of 1.5mM, we observed the pink-reddish color expected for the production of lycopene by the bacteria containing the plasmids in **C** and **D**, as a result of the synthesis of the genes (*crtE* + *crtB* + *crtI*) that encoded for this carotenoid. Furthermore, by observing the colors of the tubes when the concentration of



**Fig. 7.** Results of the extraction of Lycopene and  $\beta$ -Carotene from bacteria *E. coli* MG1655. **Up:** The image shown on the left contains the picture taken to the pellets obtained after the extraction of the carotenoids by acetone. The table displayed on the right contains the information regarding the combinations of the plasmids that were used in the double transformation of the bacteria *E. coli* MG1655 to produce the carotenoids, they were labeled as **A, B, C, D, E, F**. **Down:** The table displayed is a zoom in the results of picture taken to the pellets obtained along with the amount of the concentration of IPTG and arabinose that was added to the growth media of the double transformed bacteria *E. coli* MG1655.

arabinose raised up to 15mM, we noted how the intensity in the pink color decreases. This last result suggests on one hand that perhaps the high concentration of arabinose is somehow toxic for the cells when there is not IPTG. On the other hand, the decrease could have been due to a wrong manipulation of the plasmids or the inducers while the experiment was carrying on, because when IPTG was present and the concentration of arabinose was increased, we noted that the production of lycopene increased for the results obtained in the drop assay analysis. Furthermore, we observed the highest intensity of red color for a concentration of 1mM of IPTG and 15mM of arabinose.

Besides, the results of the production of  $\beta$ -carotene via *E. coli* MG1655 transformed with the plasmids contained in **E** and **F** (encoding for the synthesis of the genes *crtE* + *crtB* + *crtI* + *crtY*), are displayed in the rows **E** and **F** of Figure 7 **Down**. For instance, we were expecting to observe a range of light and dark orange depending of the concentration of arabinose and IPTG. However, few cases obtained did not satisfy this condition. The observations

from the figure 7 leads to the following arguments:

- In absence of IPTG and arabinose, we observed a light orange color presented in the tubes, and even darker by considering the result with the plasmids in **F**, which is an expected result due to the leakiness of the promoters pBad and pT5.

- In absence of IPTG and 1.5mM of arabinose, the production of  $\beta$ -carotene by bacteria with the plasmids in **E** reflected a very light yellow color instead of slightly darker orange color, compared to the one obtain with 0M arabinose. Besides, by using the plasmids in **F** the color of the production reflected it is not exactly orange, it looks more similar to a light pink color instead of orange, but at first glance it is not easy to give a reliable conclusion. These results could be due to a wrong manipulation with the addition of the inducers and (or) with the plasmids introduced.

- We observed an orange color in the results with the plasmids in **E** when the concentration of arabinose was 15mM with 0mM IPTG, which indicates that the genes for the production of  $\beta$ -carotene in the plasmid pSB2K3\_pBad\_CrtEBIY were expressed, but in the case of **F** we saw a pink color, which suggested that instead of the plasmid for the  $\beta$ -carotene production, perhaps pSB2K3\_pBad\_CrtEBI was introduced. The same results was obtain when arabinose was 1.5m and IPTG 1mM, where it is visible a pink color in the production.

- We were not able to discriminate any production of  $\beta$ -carotene when the concentration of arabinose was 15mM with 100 $\mu$ M IPTG, and 0M, 1.5mM with 1mM IPTG. Nevertheless, we observed orange colors for the cases when arabinose was 1.5mM with 100 $\mu$ M IPTG, where it is clear a darker orange present in the results with the plasmids in **F**, in comparison with the orange observed with the plasmids in **E**, which indicates that an increment in the induction of the promoter pT5, increased the expression of the genes *idi* and *dxs* and indeed it increased the production of  $\beta$ -carotene.

Even though we were able to discriminate the synthesis of lycopene and  $\beta$ -carotene from the engineered *E. coli* by observing the colors in the respective tubes shown in Figure 7, we found difficult to give a conclusion regarding the highest production for some of the outcomes, due to the fact that the colors seemed to be the same at first glance. To obtain a better discrimination for the production of these compound, we measured the absorbance for each of the results via a spectrophotometer. However, in order to compare the concentration of carotenoids produced in bacteria *E. coli*, we took into account the number of cells present. Thus, we measured the optical density OD<sub>600nm</sub>, in order to normalize the data obtained from the absorbance to acquire a better discrimination among the samples.

In Figure 9 are displayed the results obtained by the absorbance as a function of the wavelength for each production of lycopene and  $\beta$ -carotene respectively at different

concentrations of arabinose and IPTG.

To proceed, we made the following analysis. Accordingly to our knowledge in optics, we knew that the spectrophotometer is able to measure the absorbance by computing the intensity of the light that is able to pass the sample ( $I_{out}$ ) and compare it with the initial intensity of that light ( $I_{in}$ ), i.e.

$$A = -\log \left( \frac{I_{out}}{I_{in}} \right) = cde \quad [1]$$

Where  $c$  represents the concentration,  $d$  the path length and  $\epsilon$  the extinction coefficient. This last equation means that as the highest the absorbance is, the less is the light that is able to pass through the sample and the value of the concentration increases. On the other way around, a low absorbance implies a sample containing a less concentration.

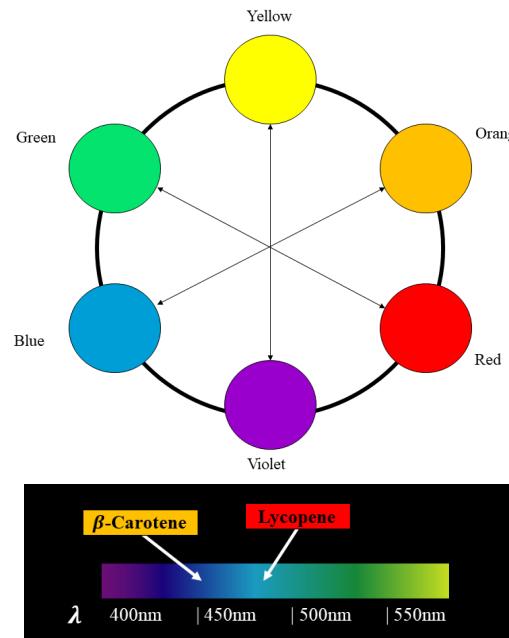
Now, when a compound shows a specific color, as in the case of orange for the  $\beta$ -carotene or red for lycopene, this means that the molecules that conform it are absorbing the visible light and reflecting the corresponding wavelength for the color that is observed. In particular, the relation between the complementary colors allows us to have an idea regarding the expected color to observed when the wavelength of the highest absorption for an element or compound is known. For instance, as is displayed in figure 8 Up, a compound that absorbs at a high level the blue light will reflect back its opposite color (orange), or if it absorbs more of green wavelength compared to the other wavelengths, this compound will reflect back red. So in theory, for  $\beta$ -carotene, the respective wavelength with the highest absorption is  $\lambda_{nm} = 451nm$  with an extinction coefficient of  $\epsilon = 139500M^{-1}cm^1$ , and for lycopene  $\lambda_{nm} = 474nm$  with an extinction coefficient of  $\epsilon = 150479M^{-1}cm^1$  (Figure 8 Down).

So, the Absorbance/OD<sub>600nm</sub> as a function of the wavelength for each of the different transformations **C**, **D**, **E** and **D**, allowed us to identify what is the wavelength with the highest absorption for each sample at different concentrations of arabinose and IPTG to determine if the production was lycopene or  $\beta$ -carotene.

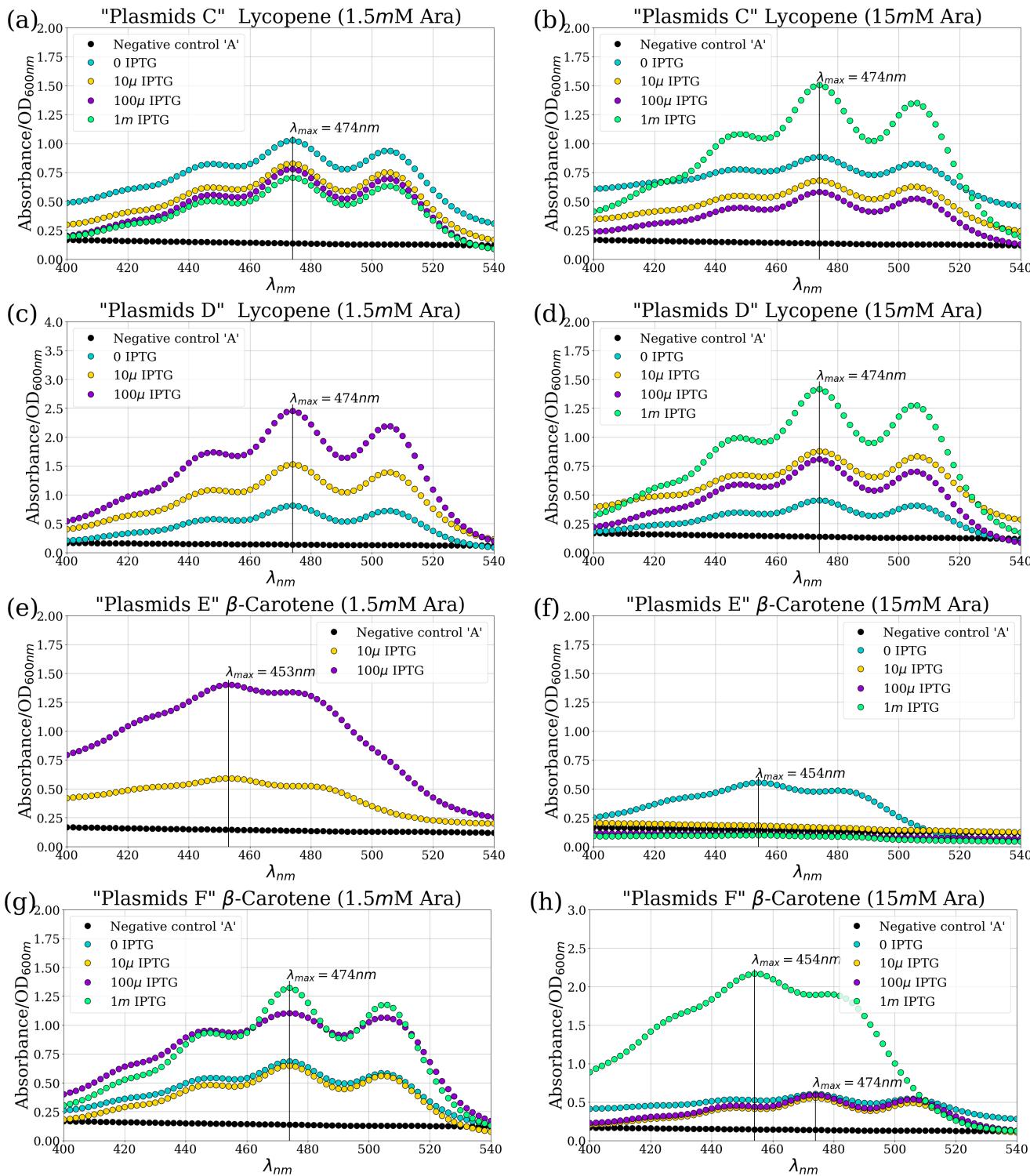
In the case of production of the cells transformed with the plasmids in **C** illustrated in graphs (a) and (b) of Figure 9, we found a maximum for  $\lambda_{nm}$  at 474nm for each of the 4 concentrations of IPTG with arabinose at 1.5mM and 15mM. In the case of the graph (a), the highest absorbance was obtained when the concentration of IPTG was 0 M and it decreased when the concentration of IPTG increases. Even though this result is unexpected (because the plasmids contained in **C** do not have any IPTG inducible promoter controlling the genes for lycopene), the difference between the values of absorbance are not highly significant, and we can not conclude any specific contribution of the IPTG to the results from the cell containing the plasmids in **C**. Similarly in the graph (b), the absorbance increased when the concentration of IPTG was increased. We also noted that the increase in the concentration of arabinose in the graph (b), increased only the absorbance of the sample with 1mM of IPTG which suggest that the other samples with different concentrations of IPTG were not correctly transformed or

cultivated. In contrast, for the results of the production with the cell transformed with the plasmids contained in **D**, shown in the graphs (c) and (d) of figure 9, was obtained a maximum value of the absorbance for  $\lambda_{nm}$  at 474nm for each of the 4 concentrations of IPTG with arabinose at 1.5mM and 15mM, which suggests that is a red-pink reflection, in agreement with the lycopene production. Further, we can see clear that the increase in the concentration of IPTG, increases the absorbance of the samples, and therefore increasing the production of the lycopene due to the over-expression o the genes *idi* and *dxs* controlled by the promoter pT5.

The results of the absorption in the graphs (e) and (f), displayed in the Figure 9, for the production of the carotenoids by bacteria *E. coli* MG1655 transformed with the plasmids in **E**, showed a maximum of  $\lambda_{nm}$  at 453nm, which implies that the carotenoid produced was  $\beta$ -carotene, and it is in agreement with the observations displayed in Figure 7 Down. Also, in these results is notable a decrease in the absorbance when the concentration of arabinose increases as it was seen in the visual analysis of figure 7. Moreover, we mentioned before that it was arduous to discriminate some of the colors obtained for the transformation in **F**. Now, in the plots (g) and (h) of Figure 9, we could noted production of lycopene instead of  $\beta$ -carotene when the concentration of arabinose was 1.5mM and 15mM. This result means that the plasmid pSB2K3\_pBad\_CrtEBI was inserted rather than pSB2K3\_pBad\_CrtEBIY, except in the case with Ara



**Fig. 8. Up:** Chromatic circle illustrating the complementary colors linked by the arrows. If an element or compound absorb at high amount any specific wavelength, its opposite color will dominate and for instance will be reflected. **Down:** Illustration of the wavelength absorbed by  $\beta$ -Carotene and Lycopene in the visible electromagnetic spectrum.

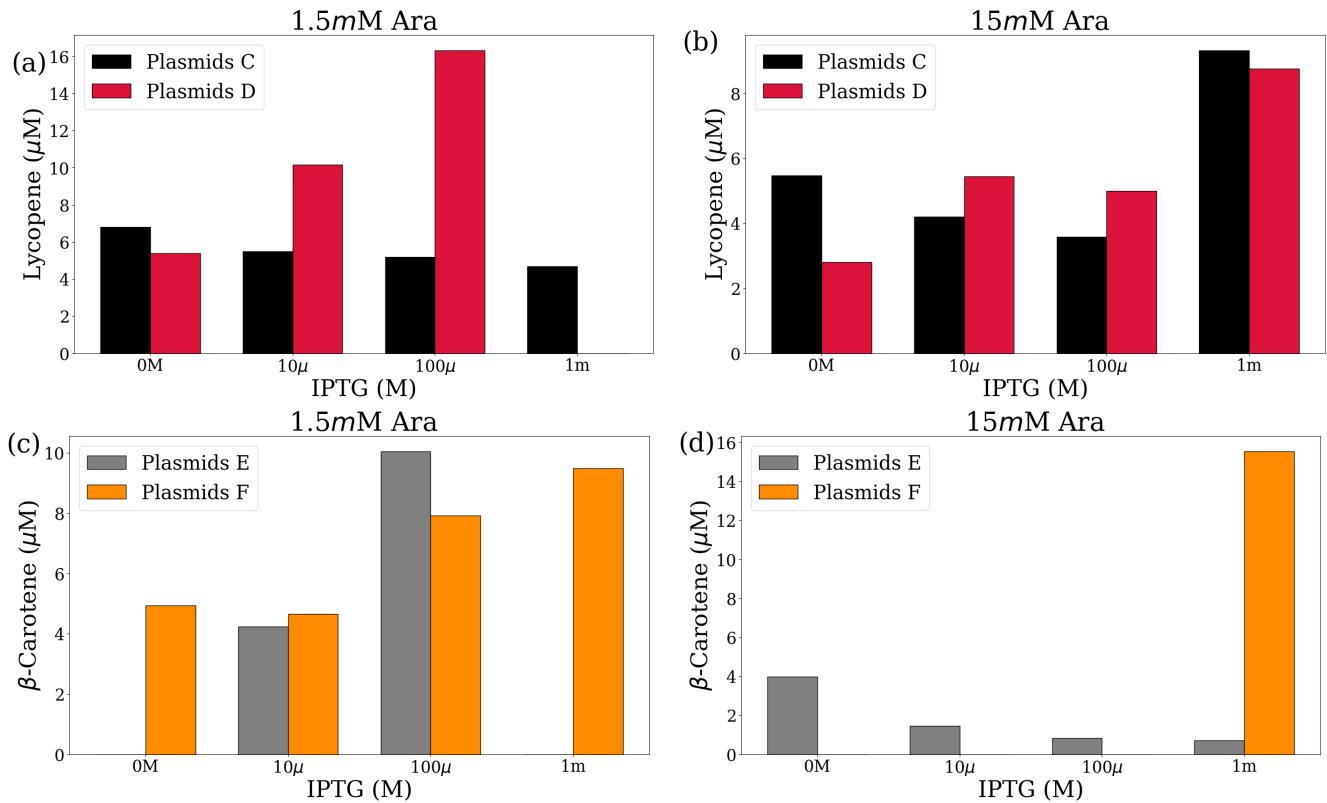


**Fig. 9.** Quantitative measurement of the absorbance to determine the production of Lycopene and  $\alpha/\beta$ -Carotene in LB media. Each plot contains information about the normalized absorption for each of the double transformation with the plasmids in **C, D, E, F** as a function of the wavelength  $\lambda_{nm}$ .

at 15mM and IPTG at 1mM.

Once we discriminated if the production was lycopene or  $\beta$ -carotene by the bacteria *E. coli* containing each of the plasmids combinations in **C, D, E, F**, we proceed to compare the amount of the production by computing the

concentration applying the formula 1 and solving for  $c$ . These results are displayed in the Figure 10, where it was created a bar plot for lycopene and  $\beta$ -carotene for each of the concentrations of arabinose (1.5mM and 15mM), where it is pointed out inside each plot the different con-



**Fig. 10.** Lycopene and  $\beta$ -Carotene final product concentration from bacteria *E. coli* MG1655 transformed with the plasmids in **C, D, E, F**, cultivated in LB media Luria/Miller at different concentrations of IPTG and arabinose.

centrations of IPTG.

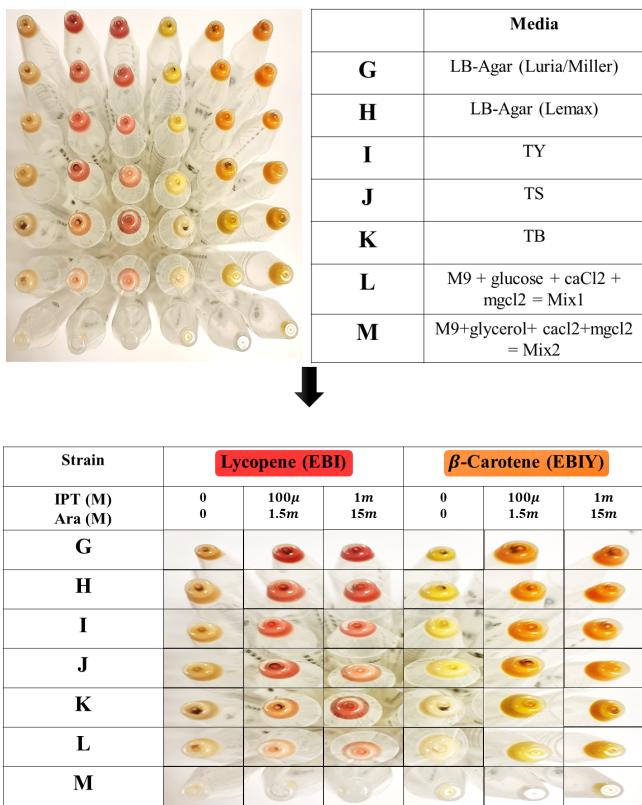
In the plots of (a) and (b) in Figure 10 are displayed the results of the concentrations of lycopene obtained. In the plot (a), for arabinose at 15mM, we perceived that the increased in the concentration of lycopene was significant for the production in bacteria transformed with the plasmids in **D**, and the highest production was obtained when IPTG was 100 $\mu$  with a lycopene concentration of 16.3 $\mu\text{M}$ . In contrast, for the plot (b) with arabinose at 15mM, the highest production (10.0 $\mu\text{M}$ ) was obtained by the bacteria transformed with the plasmids in **C** with IPTG at 1mM despite of the fact that in one of the plasmids contained in **D** was encoded the information for the over-expression of the genes *idi* and *dxs*.

Finally, in the plots (c) and (d) in Figure 10 are displayed the respective concentrations obtained for the production of  $\beta$ -carotene by *E. coli* MG1655. In the plot (c) for a concentration of arabinose at 1.5mM, the highest production (10.0 $\mu\text{M}$ ) was obtained by bacteria transformed with the plasmids in **E** with IPTG at 100 $\mu$ M. In contrast, we found that the bacteria transformed with the plasmids in **F** give the maximum amount of the concentration of the  $\beta$ -carotenoids (15.5 $\mu\text{M}$ ) with the maximum value of the IPTG (1mM).

#### Carotenoids production in various media

In the previous results, we were interesting in obtaining  $\beta$ -carotene and lycopene from bacteria *E. coli* MG1655 and to compare among different samples the highest production. Everything done before was performed by cultivating the bacteria in a constant LB media (specifically Luria/Miller). However, we realized that there are several kind of LB media that can be used for the growth of bacteria, and some of them can be more suitable than others due to their nutritional content. For instance, the goal in this section is to show the results of the production of carotenoids in seven different LB media at different concentrations of the arabinose and IPTG to discriminate the optimal media for the bacteria to growth to achieve the highest production.

We cultivated the cell already transformed with the plasmids in **D** and **F**, which contained the combinations of the plasmids pSB2K3\_pBad\_CrtEBI + pSB1A3GB3\_pT5\_idi\_dxs (encoding for lycopene) and pSB2K3\_pBad\_CrtEBIY + pSB1A3GB3\_pT5\_idi\_dxs (encoding for  $\beta$ -carotene), in seven different whose description is pointed out in the table of figure 11 Up right. Then, by the extraction of the carotenoids with acetone, we obtained the results displayed in figure 11 Down, where is also shown the different concentrations for each of the inducers added to the media. We observed production of the carotenoids in all the media used except in the media **M**. The absence of growth in this case is prob-



**Fig. 11.** Results of the production of Lycopene and  $\beta$ -Carotene in *E. coli* MG1655 cultivated in seven different LB media at different concentration of IPTG and arabinose. **Up:** On the left is displayed the results obtained in the tubes for each sample. On the right is shown a table that contains information regarding the different LB media used to growth the transformed bacteria. **Down:** The results from the pellets are pointed out with its respective concentration of arabinose and IPTG where is also written the transformed strains for **Lycopene** (plasmid pSB2K3\_pBad\_CrtEBI + pSB1A3GB3\_pT5\_idi\_dxs) and  **$\beta$ -Carotene** (plasmid pSB2K3\_pBad\_CrtEBIY + pSB1A3GB3\_pT5\_idi\_dxs)

ably due to the replacement of glucose to glycerol, with a concentration not too suitable for the bacteria. In general, glycerol acts similar to salt in the water, which makes the bacteria to loose water from inside, or in other words, dehydrating the bacteria. So, **M** media does not represent the best scenario for bacteria to growth, it rather could kill them all. Furthermore, we noted that the increased in the concentration of the inducers, increased the concentration of the produced carotenoids in each of the six media. Finally, we noted production (slight color) of the carotenoids in absence of the inducers due to the leakiness of the promoters pBad and pt5 present in the plasmids of **D** and **F**.

Even though we observed that the intensity of the colors were higher when the concentration of IPTG and arabinose were maximum in table 11 **Down**, the differences in the colors was difficult to determined at first glance. For instance, we performed a quantitative analysis of the absorption (normalized by the OD<sub>600nm</sub>) as a

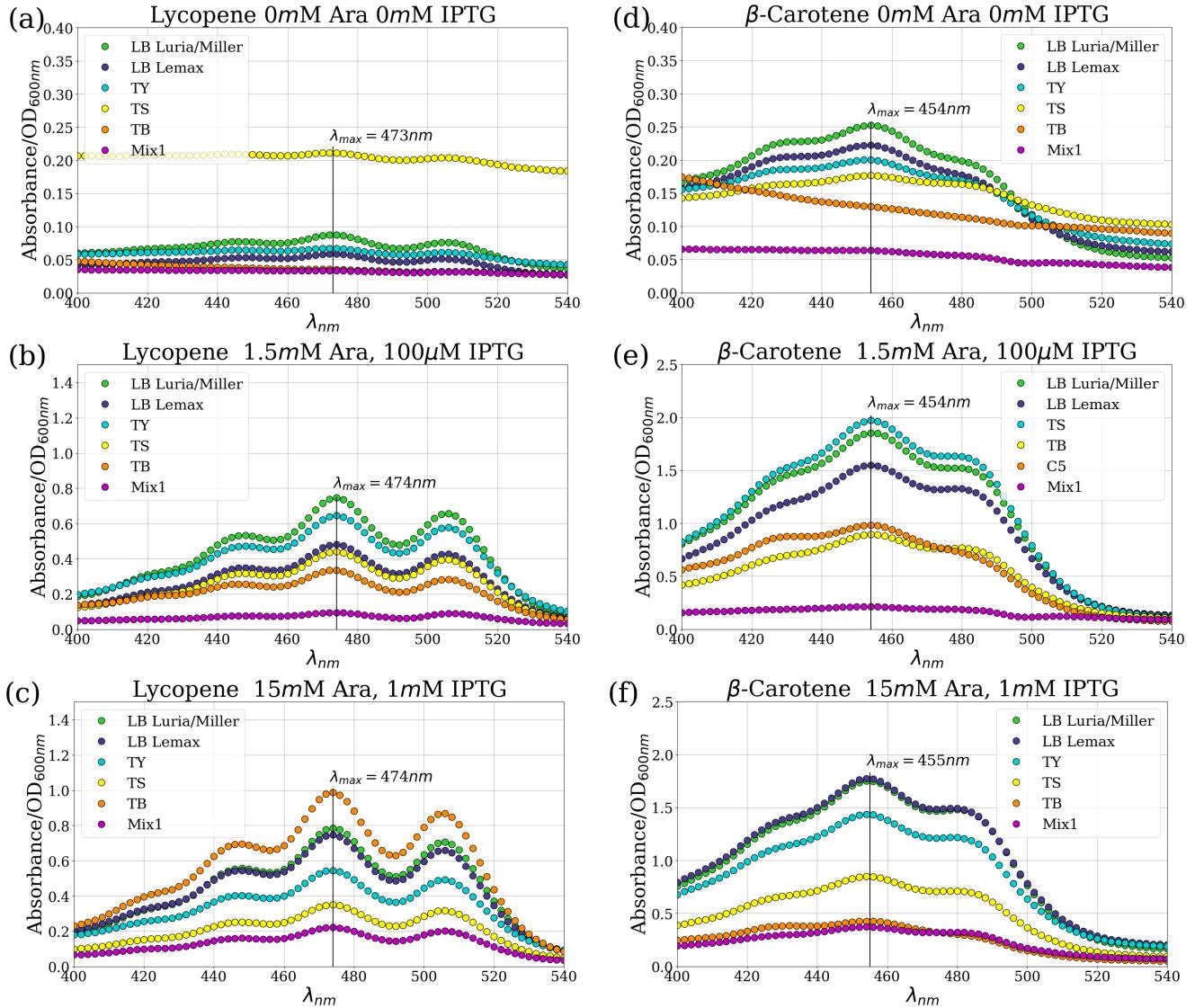
function of the wavelength in order to confirm the production of lycopene and  $\beta$ -carotene by looking the respective wavelength ( $\lambda_{nm}$ ) for the highest absorption to then with this information compute the amount of production.

The results of the absorbance for production by the engineered bacteria containing the genes necessary for the production of lycopene are displayed in the graphs (a), (b) and (c) of figure 12. For each of the cases, the highest absorbance was reach at values of  $\lambda_{nm}$  approximate to 474nm, which confirms the production of lycopene. Similarly each of the results for the absorbance as a function of the wavelength  $\lambda_{nm}$  for the production of  $\beta$ -carotene are displayed in the Figure 12, where it is shown value of  $\lambda_{nm}$  approximate to 451nm for the highest absorption.

We noted that in absence of IPTG and arabinose, we still observed production for each of the six media due to the leakiness in the promoters pBad and pT5 present in the plasmid for lycopene and  $\beta$ -carotene (graphs (a) in Figure 12 and (d) 12) as it was seen previously in the visual analysis. For the concentration of arabinose at 1.5mM and IPTG at 100 $\mu$ M, in the graph (c) of Figure 12, we found that **LB Luria/Miller** was the media with the highest absorption of lycopene (for instance highest production). In contrast, at the same condition for the concentration of the inducers, the **Tryptone Yeast Extract (TY)** was the optimal media for the production of  $\beta$ -carotene. Nevertheless, the differences between the two curves for each of these media is not highly significant, so we can conclude that these two media could share the same effect in the growth of the bacteria at these last mentioned concentration of IPTG and arabinose.

Moreover, for arabinose at 15mM and IPTG at 100mM, the production of lycopene and  $\beta$ -carotene did not share the same media to reach the highest absorption in the samples. For lycopene the highest absorbance was obtained when the media was **TB** (figure 12) while for  $\beta$ -Carotene, **LB Lemax** contributed with the maximum absorbance of the sample (Figure 12). These results mean that either the data where we were working on was incorrect due to a wrong manipulation at the time of the measure of the absorbance for each sample with the spectrophotometer, or we made a mistake in the experiment at the moment when we when manipulating the bacteria. An extra conclusion that could be considered is that one media can be more beneficial for the production of lycopene and another different one for the production of  $\beta$ -carotene.

At last, we computed the concentration of the lycopene and  $\beta$ -carotene, produced by the transformed bacteria *E. coli* MG1655 with the plasmids in **D** and **F**, cultivated in different media, similar as it was done in the previous section of the Carotenoids production in LB media to identify the optimal media. These results are illustrated in Figure 13 where two bar plots were made ((a) for lycopene and (b) for  $\beta$ -carotene) in order to compare the produced concentration obtained at different concentrations of the arabinose and IPTG inducers (15mM and



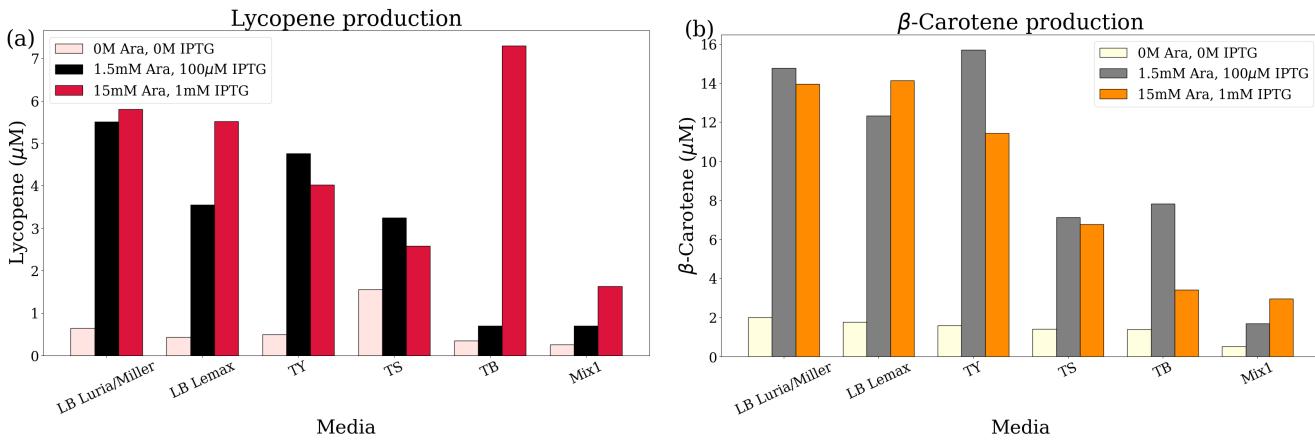
**Fig. 12.** Data analysis of the results of the absorbance as a function of the wavelength for Lycopene and  $\beta$ -Carotene produced in *E. coli* MG1655, transformed with the plasmids in D(for lycopene) and F(for  $\beta$ -carotene), which was cultivated in six different media (LB Luria/Miller, LB Lemax, TY, TS, TB, Mix1) at different concentrations of arabinose and IPTG. For each of the plots is written the wavelength  $\lambda_{nm}$  for the maximum value of the absorbance. Note that if  $\lambda_{nm} \approx 474nm$  the production is Lycopene and for  $\lambda_{nm} \approx 451nm$  is  $\beta$ -Carotene.

100μM respectively). For lycopene (plot (a)) we found that the highest production was 7.3μM in the **TB** media. Similarly, the highest production reached for  $\beta$ -carotene (plot (b)) was 16.3μM in the **TY** media along with a concentration of arabinose at 15mM and 100μM IPTG.

To finish this section, we must remark that despite of the fact our results allowed us to identify the optimal media to growth the bacteria for either the production of lycopene or  $\beta$ -Carotene, we have to take into account that we did not made a proper statistical analysis because we carried on the measurements of the experiment only once. For instance, we did not have enough data to compare among several realizations to give a reliable conclusion regarding the most optimal media for each of the samples.

## Conclusion

The applications in the field of metabolic engineering, allow to consider alternative procedures to produce several chemicals, fuels, pharmaceuticals, food, and medicines of high demand via living organisms which can generate less contamination residues in the process compared to ones obtained by purely chemical production. Additionally, the development in systems and synthetic biology helps to accelerates the “design-build-test” cycle of metabolic engineering(13). In particular, the interest in the bio-production of some carotenoids such as lycopene and  $\beta$ -carotene has increased due to their wide applications in various industries. In this work, taken into account some concepts of metabolic engineering and application of synthetic biology, we were able to produce  $\beta$ -carotene and



**Fig. 13.** Lycopene and  $\beta$ -Carotene final product concentration extracted from bacteria *E. coli* MG1655 transformed with the plasmids in **C** and **F** cultivated in different media with different concentrations of IPTG and arabinose.

lycopene by engineering bacteria *E. coli* MG1655, who was subjected to double transformations with different plasmids that contained the necessary genes the bacteria needed in order to modify its metabolic pathway and therefore produce the desired carotenoids. Furthermore, we optimized the production by the over-expression of the genes *idi* and *dxs* presented in the MEP pathway and the characterization of the foremost media for bacteria to growth. However, our results and conclusions require to be subjected to statistical analysis which is only possible by collecting data from several performance of the experiment to either denied or verified some of our observations.

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