Microalgal composition modulation for an effective biorefinering concept

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Master Program in Nanoscience, Materials and Processes; Chemical Technology at the Frontier, 2014-2015

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Abstract: As fossil fuel demand is constantly growing, the search for other sources to replace it has pointed out microalgae as the most promising candidates among alternative sources. Microalgae can synthesize a large variety of chemicals, which a part from biofuel, can also have their application in different industries such as biomedical or food and feed ones. Since biofuel production from microalgae is not economically viable, biorefineries are the only way to have economically profitable production from microalgae. However, biorefinery bottlenecks have to be overcame to counteract economic issues of microalgae implementation. Focused on one of those bottlenecks, our study aimed at developing a cultivation process to achieve modulation of industrial related compounds, by combination of different carbon sources as the strategy to direct microalgae metabolism. Changes on synthesis and productivity have been achieved as effects of carbon source additions. Carbon source combinations finally confirmed first steps on *Scenedesmus* production process modulation.

Introduction

In 2013, refined petroleum products world consumption was approximately 90 million of barrels per day, what meant more than 14.000 million L/day [1], being the major consumer United States with 18,89 million barrels per day and followed by the European Union and China (12,77 and 10,76 barrels per day respectively). As global population and fuel demand are continuously increasing, the search for other natural sources to replace fossil fuels has gained importance over the recent decades [2]. Most of the efforts in this area have been focused on biofuel production from alternative sources such as crops or microalgae. Being the latest the most interesting due to its advantages over conventional crops cultivation; microalgae have faster growth rates, require less arable lands and it cultivation causes less particle's emissions. Besides, the high lipidic content of microalgae, which can be more than 50% of total dry biomass [3], makes them a great raw material to be used for biofuel production. However, nowadays this production has not reached an effective commercialization due to the lack of competitiveness of biofuel prices compared to fossil duel ones. Biofuel production from microalgae is not cost.-effective since microalgae cultivation, harvesting and downstream procedures are energy-demanding steps which raise the final cost [4,5]. Although social aspects from this production are already covered, the technological and economic aspects make the sustainability of the process impossible.

Microalgae can, a part from lipids, synthesize and accumulate a large variety of chemicals, such as antioxidants, proteins, pigments, polysaccharides or vitamins, that makes them interesting for pharmaceutical, food and feed or biomedical industries [6–8]. Figure 1 shows the earning values from each compound utilization per 1000 Kg of biomass. The approximate cost

of production for this biomass amount is 400 €. These numbers show the need of a combination of different extractions and applications to finally achieve economic viability. The platforms which combine different compounds extractions from the biomass making the process sustainable are known as biorefineries.

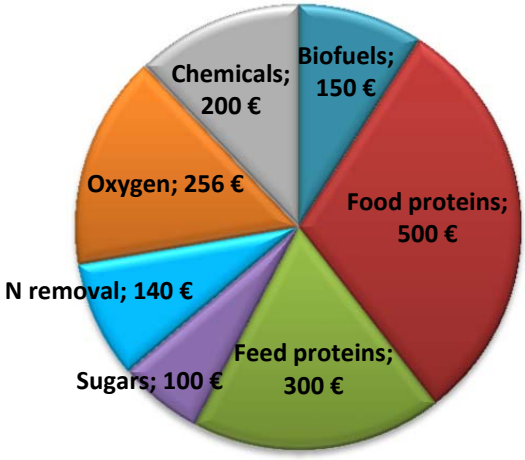

Figure 1. Value of algal biomass per 1000 Kg after biorefinering [2]

Biorefinering platforms combine different processes to produce biofuel, power and valuable co-products simultaneously to attain biofuel production costs [9]. In addition, microalgae biorefineries bring some environmental benefits that grant the process an extra added value: microalgae utilization cause lower greenhouse and climate change effect, due to microalgae's ability to mitigate CO_2. Both microalgae applications and the environmentally friendly properties that the process provides, make biorefineries potential tools for a sustainable development [10].

Nevertheless, the biorefinering process has some technological barriers. Both engineering and biochemical procedures have to be optimized to overcome those bottlenecks. Downstream procedures that allow total biomass exploitation should be studied and improved to obtain all compound fractions, as nowadays the operation units used are not able to extract one fraction without damaging the others [4]. Disruption and mild extraction techniques are being studied to improve downstream steps in microalgae biorefinering [4,11].

On the other hand, production of highly valuable co-products needs to be optimized to increase productivity and counteract biofuel economic issues.

Growth rates, quantity and quality of secondary metabolites should be parameters that must be under control to improve the whole process and its cost. Therefore, directing secondary metabolite production could be a way to balance out economic issues of microalgae implementation. Due to the huge diversity of species that have been described there is a high variability of quantity and quality of these secondary metabolites which is inherent to microalgal nature. Moreover, the different habitats where microalgae grow (they can be found in all ecosystems) also give variability to the biochemical composition. According to G. Markou (2013) [12], microalgae can vary their natural own biochemical composition when grown under stress conditions. Microalgae change their metabolism in order to maintain their growth and adapt themselves to survive to the modified conditions. Thus, secondary metabolites production changes depending on the nutrients available in the media and conditions microalgae are cultivated.

The capability of directing metabolite production would overcome one of the biorefinering bottlenecks; nowadays microalgae-based refineries require the continuous growth conditions optimization and the stop and restart of all the process to change microalgae specie for different purposes. If microalgae metabolism could be modulated, the high cost of this operation would be reduced, as changes in the cultivation media would lead to a selective chemical production, without the need of changing neither growth conditions nor used microalgae. This will also help to optimize source exploitation and to adjust the production to the market demand in a fast and cost-effective way.

The aim of the present work is to develop a cultivation process for the modulation of valuable compounds production. Using different carbon sources (glucose and sodium acetate) we want to determine the relationship between the production of industry related compounds and the carbon source consumption, as well as between production and growth phase. We analyze biochemical composition of microalgae in different growth phases to determine their productivity. In addition, combination of carbon sources is also tested looking for synergic effects between carbon sources. Microalgae selected to perform this study was *Scenedesmus sp.*, an eukaryotic algae classified as green algae. *Scenedesmus* is one of the most common species used in massive culture due to its high lipid content and biomass production [13].

Materials and Methods

Microalgae cultivation and carbon source consumption

Scenedesmus sp. selected for this study was obtained from University of Almeria (Spain) algae collection. Cells were grown in 250 mL Erlenmeyer flasks containing 100 mL of sterile water, to which 3N-BBM+V medium was added at 2% (Bold Basal Medium with 3-fold Nitrogen and Vitamins; modified (CCAP, UK)) (control medium, CTR). Cultures were maintained in periods of 16 hours light and 8 hours dark and kept under continuous agitation (150 rpm) in an incubator (Edison, USA). Temperature was kept at 21ºC constantly.

Different carbon sources were added to the control media to perform other samples. Glucose (GLC) and sodium acetate (SA) were added both at 0,1% and 2% concentration, which were established according to previous research done by the group. Combination of both carbon sources was also performed, adding the second carbon source always at 2%. This addition was done four times per sample, corresponding to four different growth stages.

All media and concentrations tested are shown in the following table:

Table 1: Distinct media and their composition.

MEDIUM	COMPOSITION
Control **(Ctrl)**	100ml sterile water + 2% 3N-BBM+V
Glucose **(GLC)**	(Ctrl) + 0,1% GLC (Ctrl) + 2% GLC
Sodium Acetate **(SA)**	(Ctrl) + 0,1% SA (Ctrl) + 2% SA
GLC + SA	[(Ctrl) + 0,1% GLC] + 2% SA [(Ctrl) + 2% GLC] + 2% SA
SA + GLC	[(Ctrl) + 0,1% SA] + 2% GLC [(Ctrl) + 2% SA] + 2% GLC

Optical density (OD) at 680 nm was measured to monitor algal growth. It was performed using a spectrophotometer (SpectraMax 340PC, USA) in 96 well plates. Four different absorbance points were stablished coinciding with four different growth phases: lag phase or point A, exponential phase or point B, end of exponential phase or point C, and stationary phase or point D. Samples were collected at these four stages and required pre-treatment that was performed as follows: fresh microalgae biomass (10 mL, per duplicate) was transferred into 15 mL tubes and centrifuged (15 min, 5.000 rpm) (MPW, Biogen Cientifica, Spain). The aqueous layer and the pellet were kept separately at -20ºC. Supernatant was used for carbon source detection, while the pellet was used for the extractions and quantifications.

Aqueous layer was used for the quantification of carbon source. Glucose was detected by the enzymatic kit Glucose (HK) Assay Kit (Sigma-Aldrich, USA) and sodium acetate was quantified by HPLC.

Extractions and determinations

Freeze-drying process (Labcono Corporation, USA) was applied to sample pellets as treatment before applying the corresponding extraction protocol.

For lipid extractions the direct transesterification method was followed. Briefly, 0,5mL of 1,5M sodium methoxide (Sigma-Aldrich, USA) were added to lyophilized sample. Then, sample was incubated at 55ºC during 30 minutes. Next, 0,5 mL of 1,25M methanolic HCL (Fluka Analytical, Switzerland) were added. Final pH was measured with reactive strips (Machery-Nagel, Germany); when needed, methanolic HCl was added until achieving the desired pH value (pH = 1). Incubation process was done at 55ºC for 45 minutes. Sample was cooled at room temperature and 0,5mL of heptane (Sigma-Aldrich, Spain) and 2mL of saturated NaCl (Scharlau, Spain) were added to the samples. The sample was gently stirred for 30 seconds, and the upper phase was transferred to a vial for chromatographic analysis.

Determinations with gas chromatography (GC) (Varian CP-3800, USA) were carried out under conditions shown in table 2:

Table 2: Gas chromatography conditions.

COLUMN	SUPERCOLWAX10 (30m, 0,25mm, 0,25um)
FURNACE	
- INITIAL	150ºC, hold 1 min.
- RAMP 1	50ºC, hold 2 min.
- RAMP 2	250ºC, rate of 10ºC/min, hold 10 min.
GAS CARRIER	Hydrogen
INJECTOR	1µl, 250ºC, hold 1min. Split ratio of 25:1.
DETECTOR	FID, 260ºC

All analyses were performed by duplicate. Resultant chromatograms were compared to pattern chromatogram [14], in order to identify each peak.

Proteins from the samples were extracted following Barbarino & Lourenço protocol [15] with some modifications. Briefly, 4 mL of ultra-pure water (MiliQ water system, Millipore, USA) were added to lyophilized sample for overnight incubation at 4ºC. After incubation, cells were disrupted using Mini-Bead Beater (BioSpec Products, USA) with 0,5 mm zirconium/silicon pearls (BioSpec Products, USA) for 3 minutes at medium speed. The suspension was centrifuged at 4ºC during 10 minutes at 15.000 rpm (Eppendorf 5417R, Germany). 1 mL of cold TCA 25% (w/v) (Scharlau, Spain) was added to precipitate proteins which were collected in a new tube. Disrupted samples with TCA were kept on ice for 30 minutes and centrifuged again at 4ºC at 15.000 rpm, during 15 minutes. Supernatant was discarded and 1 mL cold TCA 10% (w/v) was added to the pellet. The sample was centrifuged at the same conditions and the supernatant was again discarded. Then, 1 mL of cold TCA 5% (w/v) was added to the pellet to solubilize proteins and a final centrifugation was performed (15 minutes, 15.000 rpm, 4ºC). Finally, after discarding the supernatant, pellet could be kept at -20ºC until further analysis, or resuspended with 1 mL of SDS 1% (w/v) (Scharlau, Spain) for its immediate analysis.

Protein determination was carried out following instructions provided by Micro BCA™ Protein Assay Kit (Thermo scientific, USA). Previous standard sample preparation and requirements for incubation and reagents are appropriately described in the kit.

Carotenes were extracted by adding 0,5 mL of methanol 96% (v/v)(Scharlau, Spain) and the sample was homogenized with Mini-Bead Beater (BioSpec Products, USA) with 0,5 mm zirconim/silicon pearls (BioSpec Products, USA) for 1 minute at medium speed. The mixture was centrifuged twice at 15.000 rpm during 10 minutes, collecting the supernatant layer. Finally, the last supernatant collected was transferred to a new Eppendorf tube and absorbance was measured by triplicate in a spectrophotometer (SpectraMax 340PC, USA) at 470, 653 and 666 nm to detect total carotene, chlorophyll a and chlorophyll b content, respectively. Carotenoid total content was determined using following formula [16]:

$$[\text{Chlorophyll a}] = (15{,}66 * A_{666}) - (7{,}34 * A_{653})$$

$$[\text{Chlorophyll b}] = (27{,}05 * A_{653}) - (11{,}21 * A_{666})$$

$$[\text{Total carotene}] = (1000 * A_{470}) - (2{,}86 * \text{Chl a}) - ((129{,}2 * \text{Chl b})/245)$$

Results and Discussion

Protein, carotene and lipid contents were analyzed in all samples at four different growth phases, as described in materials and methods section. Both protein and carotene quantifications from CTR showed the same trend: a decrease during the lag phase, but once the exponential phase was achieved, they were increased, raising the maximum contents after 37 days (for proteins; point C) and after 44 days (for carotenes; point D). Results obtained with CTR samples were used as reference for the rest of analyses performed. The maximums levels of each compound were stablished as the baselines which the rest of the results were compared to. (Figure 2-I,II,III and IV). Fatty acids (FA) present on CTR were also identified and used as a reference.

In GLC samples neither proteins nor carotenes contents were enhanced when compared to the stablished baselines. Results showed an inverse correlation between glucose concentration added and final content of proteins and carotenes (Figure 2-I and 2-III). This decrease in carotene synthesis was expected, as in mixotrophic culture light is not the sole energy source, so energy can be obtained from the added sugar (glucose) decreasing the need of obtaining energy from the light. Therefore, less photosynthetic pigments are synthesized [17], as pigments are responsible for capturing light to be used in photosynthesis.

On the other hand, SA samples showed a different behavior in terms of production; proteins were highly increased in both concentrations tested, reaching values above 50% and 100% compared to the control (Figure 2-II), while carotenes were significantly enhanced at 0,1%

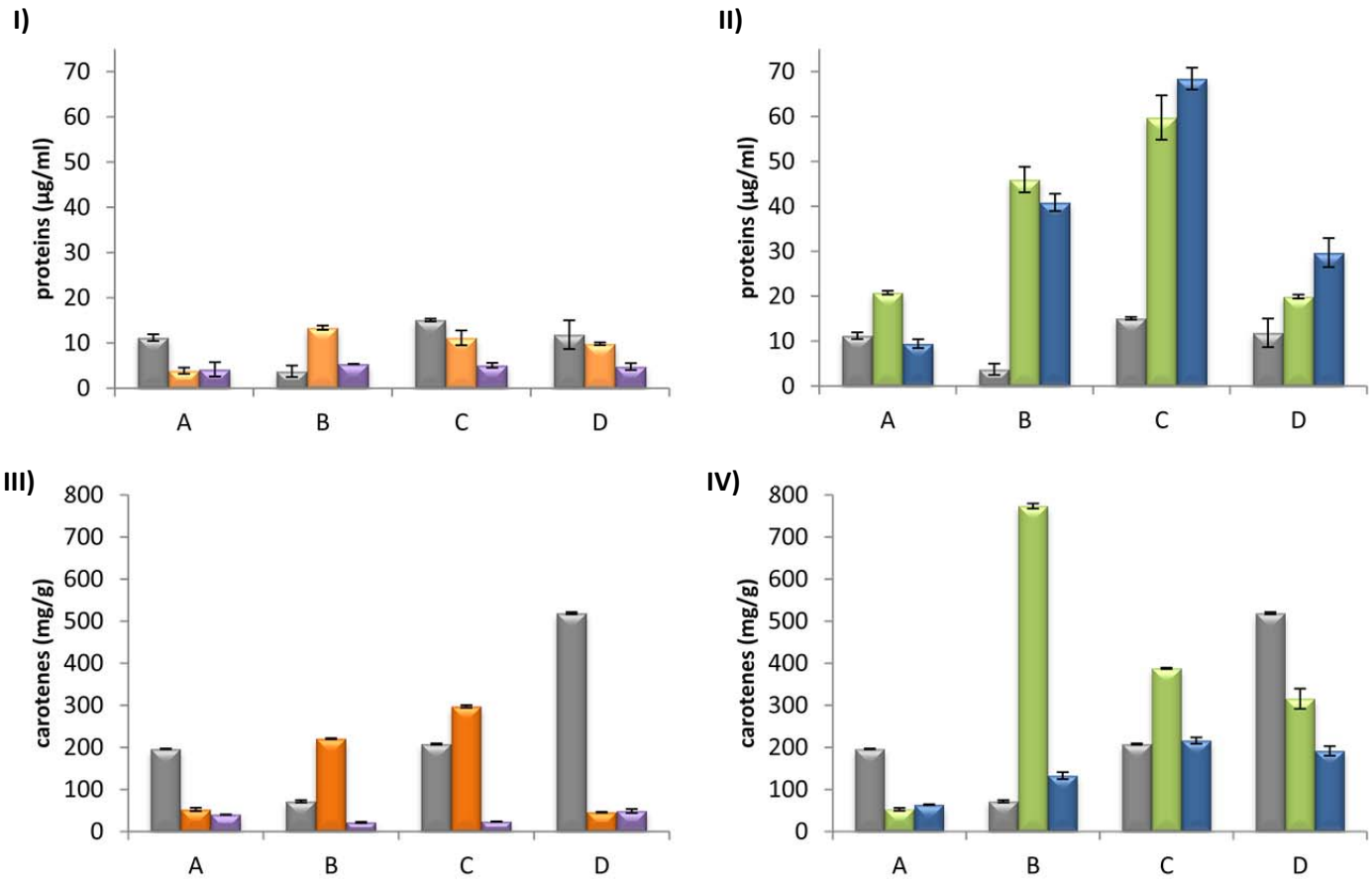

Figure 2. Carotene quantification (mg/g) from CTR, GLC **(I)** and SA **(II)**. Protein quantification (µg/mL) from CTR, GLC **(III)** and SA **(IV)**. X axis indicates four different growth phases: A (lag phase), B (exponential phase), C (end of exponential phase) and D (stationary phase). Ctrl sample 0,1% GLC 2% GLC 0,1% SA 2% SA.

concentration (Figure 2-IV). The same behavior was also found in other studies performed with different microalgae species, such as *Chlamydomons globosa, Chlorella minutissima, Spirulina platensis* or *Scenedesmus bijuga,* when acetate was added to the media [18,19]. In these studies total pigment synthesis was increased more than the 50% compared to the photoautotrophic reference culture.

Carbon source consumption was monitored. Both GLC and SA were depleted in the exponential phase (Data not shown). These results suggest that the changes among the samples are due to the addition of the carbon sources, as no other parameters that affect microalgal behavior were changed (light, temperature or pH).

Lipid analyses showed an increased FA synthesis in samples were a carbon source was added. In general, almost all lipids present in GLC samples were also present in the CTR. Most abundant lipids found in GLC samples were palmitoleic (C16:1) and heptadecanoic (C17:0), which were enhanced in both concentrations tested. Values were 7 and 11 times higher than the control in 0,1% sample, and 8 and 6 times higher in 2% for both FA respectively (Table 4). Palmitoleic acid is commonly extracted from sea buckthorn (*Hippophae rhamnoids*) and from macadamia nut the (*Macadamia integrifolia*) [20] and heptadecanoic acid is widely found in nature (from bacteria to higher plants) but is usually present as a minor FA [21]. As those fatty acids are commonly used in combustion of diesel engines, microalgae could become another important source. In SA samples, the highest amount of lipids found corresponded to palmitic fatty acid (C16:0), which was increased by 10 and 12 times compared to the control baseline (Table 4). Palmitic acid is used in cosmetics and food industries, and high quantities of this fatty acid are also useful for biofuel production. This finding opens a new possibility for fatty acid production for cosmetic and food purposes where microalgae might replace traditional or mutant crops in these industries. Moreover, acetate is a readily available and inexpensive substrate derived from many industrial applications [22,23].

Most increased fatty acids from analyzed samples are listed below. In table 2 all results were normalized using control sample's results as baseline (**x**), and the numbers before the x indicates the relative amount of this fatty acid compared to the reference (CTR).

Table 4: Increased fatty acids at different growth phases in glucose and sodium acetate samples

	GLC samples				**SA samples**			
	0,1%		**2%**		**0,1%**		**2%**	
	B	**C**	**B**	**C**	**B**	**C**	**B**	**C**
C4:0/C6:0	3,5x		1,4x		4,2x		6,6x	
C16:0	5,4x		1,5x		10x		12x	
C16:1	4,2x	7,6x	2,2x	8,5x	1,2x		4x	1,7x
C17:0		11x		6,5x		5,6x		7x
C17:1	3,2x	4x	x	1,3x	2x	2,6x	3,4x	1,8x
C18:2n6t/ C18:2n6c	1,8x		1,6x		3,7x		4,2x	
C18:3n6	3x	2,6x	x	1,5x	2,6x	3,2x	2,7x	1,5x
C18:3n3	5x		x		3x		3,7x	

C4:0/C6:0: butyric/caproic, **C16:0**: Palmitic, **C16:1**: Palmitoleic, **C17:0**: Heptadecanoic, **C17:1**: cis-10-heptadecanoic, **C18:2n6t/C18:2n6c** : Linoelaidic/linoleic, **C18:3n6**: ɣ-linoleic, **C18:3n3**: linolenic.

Results of total production indicated that glucose addition is useful for fast biomass obtaining, while sodium acetate is the best carbon source tested to enhance protein and carotene quantities. Due to the availability and the low cost of this carbon source, sodium acetate is a good candidate to be used in industries where high content of proteins or carotenes are needed. Moreover, SA brings an extra value for its utilization: palmitic acid is greatly increased with its addition and elevated concentrations of this fatty acid are desired for biodiesel production [22]. Therefore, sodium acetate is pointed out as a sustainable

carbon source for its use in microalgae-based biorefineries allowing more efficient biomass exploitation.

Time is an important element to consider when scale-up is done at industrial level. To introduce time element to our study, we calculated the productivity in the different analyzed growth phases. Productivity was calculated as the compound amount obtained in each growth phase, divided by the number of days that it took to reach that growth phase (concentration/day). Although total production was low in most cases, productivity is high when compared to control, because carbon source addition to media accelerated *Scenedesmus* growth.

The best productivity in CTR sample was reached after 44 days, where protein production was 0,41 (µg/ml)/day and carotene one was 11,5 (mg/g)/day. Although GLC samples did not provide important results when compared to the maximum in the CTR, they showed incremented productivity when compared to CTR baselines. For instance, in both glucose concentrations, protein productivity was almost doubled (0,71 (µg/ml)/day for 0,1% sample and 0,76 (µg/ml)/day for 2%) at point B, corresponding to the beginning of the exponential phase. However, carotene analyses did not show high productivity rates when compared to the CTR. As abovementioned, low carotene productivities were expected in GLC samples, as results from production did not differ significantly from the baselines. SA samples showed a high productivity of proteins, reaching the highest productivity at the exponential phase (point B), with values of 3,06 and 4,54 (µg/ml)/day at 0,1% and 2% concentrations respectively. Carotene highest productivities were also found at point B, reaching values of 51,57 (mg/g)/day and 14,76(mg/g)/day (0,1% and 2% concentration) compared to the control. Point B was reached in less than 15 days in samples grown with sodium acetate and showed the best productivity results. Both protein and carotene productivities were enhanced at the same point, making the use of sodium acetate very interesting because at industrial level, it would imply more efficiency of the production system, and it finally means final cost reduction.

We have demonstrated the effect of carbon source addition to change microalgal component synthesis and productivity. Consequently, next step is to study the modulation effect of these carbon sources. To test this modulation we performed combinations of the carbon sources (GLC + SA and SA + GLC), with two concentrations (0,1% and 2%) and at four different growth phases (A, B, C and D) as explained in materials and methods section. When the second carbon source was added, independently of the growth phase, a change in the production was detected. When sodium acetate was added to GLC, an increase of both protein and carotene content was observed. On the other hand, the addition of glucose to SA decreased both contents. In general, production modulation was greatly achieved in initial samples grown at 0,1%, where the addition of the second source at 2% changed significantly sample's behavior. On the contrary, initial samples grown at 2% did not showed modulation capacity. This is probably because the primary carbon source at higher concentrations was not totally consumed or metabolized when the second source was added. Thus, the effect of the second carbon source cannot be appreciated.

Conclusions

Glucose addition provided faster biomass production than the control and doubled protein productivity at low glucose concentrations. Sodium acetate showed increased production and productivity, raising values above the control ones at both concentrations tested. Therefore, the use of these carbon sources at industrial

level may allow control selective compound production. Moreover, since time represents a decisive element that affects productivity and production costs, incremented productivities help to reduce final costs.

In addition, for the first time, modulation of compound production process has been achieved by carbon source combination, providing a first step towards an effective industrial application. Modulation capability provides another economic benefit: cultivation and harvesting procedures would not change for each desired product, so those expenses would be also reduced and process can be adjusted to the market demand. Nevertheless, more work should be done before its application and scale-up studies should be performed.

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