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Analysis of iron and nitrogen interplay in lipids accumulation in microalgae

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5. Espécies reativas de oxigênio 6. CCRD I. Título

RESUMO

Análise da interação ferro e nitrogênio no acúmulo de lipídios em microalgas

O aumento da população mundial, a emissão de gases de efeito estufa e a demanda global de energia tem levado à busca por uma fonte de energia sustentável e renovável. As microalgas têm sido reconhecidas como uma fonte prospectiva de produção de biocombustíveis devido à capacidade de algumas espécies de produzir grande quantidade de lipídios neutros, como triacilgliceróis (TAGs), sob condições de estresse nutricional, de luz, pH, temperatura e salinidade ou estresses bióticos. Um dos estresses nutricionais mais conhecido e que tem chamado atenção é a privação de nitrogênio, que é conhecido por desencadear o acúmulo de TAGs dentro da célula da microalga. No entanto, essa condição de estresse afeta negativamente o crescimento celular e, conseqüentemente, diminui a aplicabilidade das microalgas como matéria-prima de bioenergia e lipídios para fins biotecnológicos, como a produção de biocombustíveis. A suplementação de nutrientes na cultura é uma abordagem que tem sido utilizada para melhorar o crescimento e a biomassa das células de microalgas. Como foi demonstrado, com a suplementação de ferro o crescimento e o conteúdo lipídico foram aumentados na cultura de microalgas. No entanto, ainda não há uma compreensão abrangente do mecanismo dessa resposta celular e da função do ferro no aumento do acúmulo de TAG e do mecanismo de interação ferro e nitrogênio que parece levar à melhoria do crescimento e ao aumento do acúmulo de lipídios. Recentemente, a otimização da condição da cultura para melhor crescimento e produção de TAG tem sido um assunto muito importante, pois as microalgas se tornaram uma fonte sustentável e promissora. Portanto, para investigar as respostas celulares das microalgas à interação da suplementação de ferro e privação de nitrogênio e explorar o efeito da interação desses dois nutrientes no acúmulo e crescimento de lipídios em *Chlamydomonas reinhardtii*, um projeto rotativo composto central (CCRD) foi realizado. A proteômica baseada em espectrometria de massa tornou-se parte integrante da biologia de sistemas. Neste estudo, a análise proteômica quantitativa *shotgun* em séries temporais, realizada em diferentes condições experimentais com alteração da concentração de ferro e nitrogênio, revelou a identidade de proteínas que podem participar da interação nitrogênio e ferro na modulação do crescimento celular e no desempenho do acúmulo de TAGs na espécie modelo *C. reinhardtii*. Os resultados ilustram que a abundância de proteínas envolvidas na fotossíntese, processos de oxidação-redução e biossíntese de poliaminas foi significativamente modulada pela interação entre ferro e nitrogênio, mantendo o desempenho de crescimento das células e os níveis de lipídios elevados. Embora a manipulação de micronutrientes e macronutrientes no meio de cultura cause estresse e conseqüentemente aumente a produção de espécies reativas de oxigênio (ROS), parece que essa interação diminui os níveis de ROS dentro da célula, resultando em um equilíbrio entre crescimento e acúmulo de lipídios.

Palavras-chave: Biologia de sistemas, Triacilglicerol, Bioenergia, Biocombustível, Espécies reativas de oxigênio, CCRD

ABSTRACT

Analysis of iron and nitrogen interplay in lipids accumulation in microalgae

Increasing the world population, emission of greenhouse gasses and global energy demand has led to the quest for a sustainable and renewable source of energy. Microalgae have been identified as a prospective source of biofuel production due to a large number of microalgae species that boast the ability to produce a large amount of neutral lipids, such as triacylglycerols (TAGs), under stress such as light, pH, temperature, salt, and nutrient stress or biotic stresses. One of the most conspicuous nutrients stresses that have been accentuated is nitrogen deprivation which is known to trigger TAGs accumulation inside the microalgae cell. On the downside, this stress condition impacts the cell growth and consequently decreases microalgae's applicability as a bioenergy and lipids feedstock for biotechnological purposes such as biofuel production. Supplementing the culture with nutrients is an approach that has been utilized to improve the growth and biomass of microalgae cells. As it has been shown, with iron supplementation the growth and lipid content were enhanced in microalgae culture. However, there is still no comprehensive understanding of the in-depth mechanism of this cellular response and the function of iron in enhancing the TAG accumulation and the iron and nitrogen interaction mechanism which seems to lead toward improving growth and increasing lipid accumulation. Recently, optimizing the culture condition for better growth and TAG production has been a very important subject as microalgae became a promising sustainable source. Therefore, to investigate the cellular responses of microalgae to the interplay of iron supplementation and nitrogen deprivation and to explore the interaction effect of these two nutrients on lipid accumulation and growth on *Chlamydomonas reinhardtii*, a Central Composite Rotatable Design (CCRD) was carried out. Mass spectrometry-based proteomics has become an integral part of the systems biology. In this study, in the model species microalgae *C. reinhardtii*, mass spectrometry-based Label-free time-resolved quantitative shotgun proteomics analysis of multiple experimental conditions showed the identity of proteins that may involve in nitrogen and iron interaction in the modulation of cell growth and TAGs accumulation. The results illustrate that the abundance of proteins involved in photosynthesis, oxidation-reduction processes, and polyamine biosynthesis was significantly modulated by the interplay between iron and nitrogen while maintaining the cells' growth performance and lipid levels high. Although manipulating the micronutrients and macronutrients in the culture medium would cause stress and consequently increase the reactive oxygen species (ROS) production, it seems that this interaction could decrease the ROS levels inside the cell which results in an equilibrium between growth and lipid accumulation.

Keywords: Systems biology, Triacylglycerol, Bioenergy, Biofuel, Reactive oxygen species, CCRD

1. INTRODUCTION

1.1. The microalgae *C. reinhardtii* and its potentials for using in biofuel production

Expanding the economy and the fast growth of the population make it difficult to rely on fossil fuels as a source of energy. Increasing greenhouse gas emissions, carbon dioxide concentration, and demand for fossil fuel usage cause global warming and climate change. They are all compelling reasons for fostering the development of a new source of energy that is more environmentally friendly and sustainable. Biofuels are viable alternatives to fossil fuels in terms of renewability and sustainability. Considerable progress has been made over recent decades in biofuel production, which made the picture of the near future of using this source of energy more vivid. There are new generations of biofuels (second and third) that do not have the issue for which the previous generation was struggling. The new generations, namely lignocellulosics, microalgae, cyanobacterial and other microbes-based, do not need arable lands to be produced, which is a definite advantage (Chisti, 2007, Rodionova, et al., 2017).

Among these above-mentioned options, microalgae have several benefits over land crops, such as year-round growth and harvesting capacity, substantially higher biomass per area yield, maximum lipid content, and resistance to pests and pathogens that commonly affect the crop plants. Some microalgae have 80% oil (w/w), while the oil contents of 20-50% are commonly reported in other land plant species (Spolaore, et al., 2006; Chisti, 2007). However, how cells accumulate such amount of lipids is not completely understood. Diverse algae species have been suggested as a potential renewable biofuel feedstock because of their ability to accumulate triacylglycerides (TAGs) in the form of 'lipid bodies' under stress conditions (Wijffels and Barbosa, 2010). Among the many different microalgae species, the unicellular microalgae *C. reinhardtii* serves as a model system for studying algal physiology, including aspects of photosynthesis (Eberhard, et al., 2008), micronutrient deficiency (Hu, et al., 2008; Kropat, et al., 2011; Merchant, et al., 2012), metal accumulation (Piccapietra, et al., 2012), production of biofuel (Quinn and Davis, 2015; Rodionova, et al., 2017) and the molecular physiology of lipid accumulation (Merchant, et al., 2012). The genome of *Chlamydomonas* has been completely sequenced (Merchant, et al., 2007), and it has a developed genetic system, which makes *Chlamydomonas* easy to control in metabolic terms using gene overexpression and knock-down (Merchant, et al., 2007; Wijffels and Barbosa, 2010).

Much of the knowledge we have today about the fatty acid biosynthesis pathways and TAGs and the storage of these in microalgae cells is based mainly on genomic annotations and orthologous genes of enzymes with known function in animals, yeasts and especially in seedlings (Fan, et al., 2011; Patel, et al., 2017). However, studies carried out in the last decade, mainly with the microalgae *C. reinhardtii*, showed evidence that the synthesis of fatty acids and TAGs in these organisms presents several distinct characteristics of terrestrial plants (Boyle, et al., 2012; Zienkiewicz, et al., 2016). One difference is that, because it is unicellular, the biosynthesis of TAGs in microalgae occurs in cells that are photosynthetic, unlike plants where lipid biosynthesis occurs in greater abundance in specialized cells such as fruits and seeds (Zienkiewicz, et al., 2016). This divergence indicates that the regulation of the carbon partition within a single cell probably occurs differently from what is currently known in terrestrial plants (Hu, et al., 2008; Zienkiewicz, et al., 2016).

1.2. Induction of Triacylglycerol in the microalgae cells using macronutrients and micronutrients

Several conditions of nutrient-stress may strongly induce Triacylglycerol (TAG) production in microalgae (Bolling and Fiehn, 2005), including sulfur-stress (Fouchard, et al., 2005), phosphorus-stress (Moseley, et al., 2009), nitrogen-stress (Longworth, et al., 2012; Park, et al., 2015) and micronutrient deficiencies (Allen, et al., 2008; Haas, et al., 2009). The potential for interaction and functional crosstalk between metals and other macronutrients increases the complexity of nutritional studies (Merchant, 2010). Few detailed studies have systematically examined the combined effects of iron and nitrogen on microalgae molecular physiology and none of them deeply investigated the association of these responses to the lipid accumulation in *C. reinhardtii*. Accumulation of lipid increased in a merged effect of nitrogen deprivation, high concentration of iron and high light intensity in four strains of *Botryococcus* spp (Yeesang and Cheirsilp, 2011). Also, under nitrogen-deficient condition, the amount of TAG increased in *Neochloris oleoabundans* with an increase in iron concentrations (Sun, et al., 2014). To better understand the phenomenon in microalgae we studied the interplay of the cellular responses under varying iron and nitrogen availability; the former one, affects the physiology of the cells by inducing increased biosynthesis of TAGs under nitrogen deprivation.

The iron possesses a crucial role as micronutrient in microalgae; therefore, these organisms have multiple pathways to assimilate it under various chemical forms (Hernandez-

Torres, et al., 2016). Iron is used as a cofactor in numerous biochemical pathways and especially its ability to donate and accept electrons makes iron important in enzymes that catalyze redox reactions.

Since iron can also react with oxygen to generate cytotoxic agents, its accessibility within the cell must be under tight homeostatic control, which requires complex regulatory mechanisms (Finney and O'Halloran, 2003). Moreover, iron is a component of many proteins involved in other vital processes such as fatty acid metabolism, and amino acid biosynthesis (Glaesener, et al., 2013). In microalgae, it has not been yet well studied the effect of different concentrations of iron on lipid quantity and growth, also the biofuel production quality (Sivaramakrishnan and Incharoensakdi, 2017).

It has been shown that the iron concentration in culture medium had an impact on cell growth and lipid content of algae such as *Dunaliella viridis* (Menzyanova, et al., 2009). By decreasing the concentration of iron inside the culture, the algae cells undergo iron depletion condition; therefore, the pathways of iron uptake upregulate. In this situation, the abundance of the ferroxidase boosts that employs for iron limitation marker (Glaesener, et al., 2013).

When there is iron deprivation in the culture, the separation of the complexes of proteins related to chlorophyll emerges, and the PSI and PSII would consequently break down. (Moseley, et al 2002; Urzica, et al., 2012; Glaesener, et al., 2013). It has been shown that the iron deficiency is one of the main factors limiting marine algal biomass productivity and under certain culture conditions result in higher quantities of lipids (Liu, et al., 2008). In the microalgae *C. reinhardtii*, iron deficiency leads to accumulating TAG in the cells (Kropat, et al., 2011). In *Chlorella pyrenoidosa* the iron limitation caused lipid accumulation inside the microalgae cells (Fan, et al., 2014).

On the other hand, high iron concentration could induce TAG accumulation in microalgae too. Compared to iron-replete condition, in the iron-excess, the cells will over-accumulate iron (Long and Merchant, 2008; Terauchi, et al., 2010). It seems that since naturally, there is no excess iron, *Chlamydomonas* has not developed any pathways to send out the excess iron, based on the literature (Glaesener, et al., 2013). Iron stress may stimulate more lipid synthesis to counteract the damaging effect of reactive oxygen species. An excess of metal, including iron, can result in the formation of reactive oxygen species such as hydrogen peroxide and other oxidant compounds, which may interact with lipids (Miazek, et al., 2015). It was observed that elevated quantities of iron in the media could stimulate the amount of carbohydrate and TAG in *N. oleoabundans* HK-129 cells, which means that there

happened some upregulation in the pathways related to these two compounds (Sun, et al., 2014). The lipid content of three microalgae, *Chlamydomonas* sp., *Chlorella* sp. and *Scenedesmus* sp. was increased in response to increased environmental iron concentration (Sivaramakrishnan and Incharoensakdi, 2017). There are other reports that indicated that high iron level is conducive to lipid accumulation in *Chlamydomonas*, *Chlorella* and *Botryococcus* (Liu, et al., 2008; Yeesang and Cheirsilp, 2011). One of the compelling reasons to be mentioned for increasing the lipid quantity inside the cells when the cells are exposed to very high levels of iron is, for example, that in the microalgae *Nannochloropsis oculata* elevated levels of trace elements drive the cells to produce proteins that are related to stress response and change the metabolic pathways of constructing starch to lipid (Dou, et al., 2013). High TAG accumulation is also observed in several species of microalgae under nitrogen deprivation (Kropat, et al., 2011; Wu and Miao, 2014). However, in microalgae cells, nitrogen limitation can generate cell inactivity (Liu and Benning, 2013; Merchant, et al., 2012), followed by chloroplast breakdown, increasing TAG, and reassembling of the structures of the membrane, eventually producing lipid bodies in the cytosol and chloroplast (Fan, et al., 2011; Hu, et al., 2008). It has been argued that enhancing lipid inside the microalgae cells is a multifactorial response, as there is a highly complicated connection among increasing lipid, deficiency of nitrogen, and stress inside the cell (Johnson and Alric, 2013). Therefore, it is still necessary to carry out studies that contemplate and seek to identify and explain the complexity of these metabolic and regulatory pathways related to lipid biosynthesis.

Such studies may encourage strategies to achieve higher productivity rates through metabolic engineering (De Bhowmick, et al., 2015).

The cellular responses to interaction of iron and nitrogen and their effects on the gene expression profiling of transcription factor and transcriptional regulator genes, total proteome and regulatory proteome and metabolome analysis of the cells, have not been broadly investigated despite the fact that they have vital role in accumulation of lipids in microalgae cells. Therefore, an investigation of the interplay between both elements may reveal possible crosstalk that affects and regulates lipids accumulation and biomass production in microalgae.

1.3. Experimental design

A systematic approach for experiments to be planned and the data be analyzed to optimize the condition of experiments therefore have an objective conclusion is experimental design. Choosing the best experimental design for an experiment increase the useful

information which will be obtained and may decrease the number of experiments (Poole and Poole, 2012). It depends on the number of variables and the objectives of the experiment. In general, experimental design has some advantages such as, the variables can be analyzed at the same time not individually in separated experiments, decreasing the number of repeats and experiments and enhancing the quality of information, optimizing more than one response simultaneously, calculating and analyzing the experimental errors, using the experimental design doesn't need advance knowledge in statistics (Rodrigues and Iemma, 2014).

Box and Wilson in 1951 developed CCRD which is one of the most suitable experimental designs for discovering and optimizing the most reasonable outcome, also an essential part of response surface methodology (RSM) (Beg, et al., 2019). Therefore, in this study we used a CCRD experimental design which we had a 2^2 factorial design, including four trials under the axial conditions and three repeats at the central point.

1.4. Systems biology and omics analyses

What is system biology? There is no definitive answer to this question, as it is a rare occurrence that two scientists have the same opinion about this. One of the definitions in summary describes systems biology as an interdisciplinary study uses biology, engineering, bioinformatics, computer science and others which use a holism and give us a whole image look and not only separated parts. Having a better biological whole system, we need a tool and approach which is "OMICS" (Karahalil, 2016).

A large number of studies have applied Omics approaches to perform systemic characterization of cellular responses of *C. reinhardtii* under different nutritional conditions through the study of its transcriptome (Boyle, et al., 2012; Kropat, et al., 2011; Miller, et al., 2010), proteome and metabolome (Hsieh, et al., 2013; Lee, et al., 2012; Mettler, et al., 2014; Schmollinger, et al., 2014).

The study of proteins in a cell and their interaction is called Proteomics (Cho, 2007). It contains a wide range of technologies which establish the quantity and identity of the proteins that expressed inside the cell. It is almost impossible to study omics on microalgae without proteomics approach, as it is a technique for comprehending biological system complexity. With proteomics approach a large number of proteins would be identified in a sample, which gives us the opportunity of finding the proteins involved in lipid production

directly and indirectly which enable us to manipulate those proteins for enhancing lipid accumulation.

6. CONCLUSION

Under nutrient stress, *C. reinhardtii* cells accumulate Triacylglycerols (TAGs) while reducing their growth rate, which results in low biomass production. The findings of this study have broadened the horizons of knowledge about nutrient stress and enhancement of biomass and TAGs.

We could obtain results which indicate under nitrogen deprivation, higher concentrations of iron can improve the growth of *C. reinhardtii* cells while increase the lipid accumulation inside the cells. It was also observed that in the same concentrations of nitrogen, increasing the iron quantity makes a big difference between the cultures and changes the maximum growth rate and doubling time of the culture. Comparing extreme high and extreme low concentrations of iron in equal nitrogen quantity (low nitrogen concentration), revealed that lipid bodies formed inside the cells with extreme high concentrations of iron. In this study, we could optimize the *C. reinhardtii* cells culture under stress condition to obtain the higher lipid and good cell production. We showed that, the interplay effect of limited concentration of nitrogen and high iron amount result in an optimum condition for *C. reinhardtii* and may be applicable for other microalgae cultures.

The results of this study and especially, the proteomics data suggest that iron supplementation of cells under nitrogen depletion may protect, at least partially, the photosynthetic machinery of *C. reinhardtii* cells, avoiding substantial reduction of the abundance of the PSI and PSII proteins, even with reduction of the levels of chlorophyll biosynthesis. The proteomics evidence indicate that the combination of high iron and low nitrogen concentration may induce the activation of mechanisms related to oxidative-stress that may be protecting the cells from the increase of oxidative-stress, typically observed during nutrient deprivation. Proteins related to redox homeostasis may have a stronger influence in the redox balance of mechanisms that take place in the chloroplast or mechanisms that affect the expression of chloroplast nuclear-encoded genes. It is highly likely that some proteins related to oxidative stress triggers iron and nitrogen optimized concentration, may enhance the biomass productivity and the lipid accumulation inside the *C. reinhardtii* cells at the same time. In this study, detecting the upregulation of some antioxidant proteins such as GPX which reduces the ROS and increases the lipid production inside the cell simultaneously, would be the proof of this assumption.

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