

RAQUEL CRISTINA VIEIRA DOS SANTOS

**EVALUATION OF THE EFFECT OF GROWTH CONDITIONS ON LIPID
PRODUCTION BY *Papiliotrema laurentii* UFV-1 IN CULTURE MEDIA
CONTAINING XYLOSE**

Dissertation presented to the Universidade
Federal de Viçosa as part of the requirements of
the Agricultural Microbiology's Graduate
Program to obtain the title of *Magister Scientiae*.

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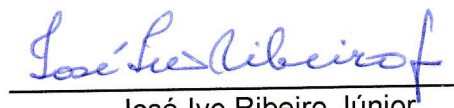
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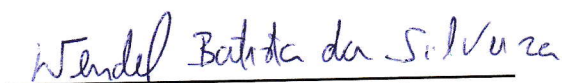
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(Co-advisor)


Wendel Batista da Silveira
(Advisor)

Aos meus pais, Nivaldo e Madalena.

À minha irmã, Rafaela.

Dedico.

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*“O correr da vida embrulha tudo.
A vida é assim: esquenta e esfria,
Aperta e daí afrouxa,
Sossega e depois desinquieta.
O que ela quer da gente é coragem”*
Guimarães Rosa

BIOGRAFIA

RAQUEL CRISTINA VIEIRA DOS SANTOS, filha de Nivaldo Ermano dos Santos e Madalena Aparecida Vieira, nasceu no dia 22 de abril de 1992, em Conselheiro Lafaiete, Minas Gerais. Graduou-se em Bioquímica pela Universidade Federal de Viçosa em julho de 2015. Em agosto do mesmo ano, iniciou o curso de mestrado no Programa de Pós-Graduação em Microbiologia Agrícola da Universidade Federal de Viçosa, Viçosa, Minas Gerais, submetendo-se à defesa de dissertação em 23 de agosto de 2017.

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ABSTRACT

DOS SANTOS, Raquel Cristina Vieira, M.Sc., Universidade Federal de Viçosa, August, 2017. **Evaluation of the effect of growth conditions on lipid production by *Papiliotrema laurentii* UFV-1 in culture media containing xylose.** Advisor: Wendel Batista da Silveira. Co-advisor: José Ivo Ribeiro Júnior

Biofuels are an alternative to reduce dependence on fossil fuels. Among them, the biodiesel produced from the transesterification reaction between an alcohol and a triacylglycerol molecule is a substitute for conventional diesel. Edible vegetable oils are currently used as a source of triacylglycerols in the biodiesel production. Nevertheless, its production from these oils competes with food production for both arable lands and water. In addition, the vegetable oil features are affected by climatic conditions. These drawbacks can be circumvented by using alternative oil sources such as microbial oil. Oleaginous yeasts accumulate at least 20% of their dry weight as lipids, mainly in the form of triacylglycerols, nevertheless, superior values can be achieved depending on the culture conditions. *Papiliotrema laurentii* UFV-1, contrary to the oleaginous yeast model *Yarrowia lipolytica*, is capable of growing and accumulating lipids from xylose, the main sugar found in hemicellulose. Therefore, this work aimed to evaluate the effects of pH, C/N ratio, agitation speed, initial cell density and temperature on lipid production by *P. laurentii* UFV-1, a new yeast strain isolated from soil. We performed the centered face central composite design, with 25 combinations between the factors studied and three replicates at the central point, to evaluate their effects on the response variable lipid content. The highest lipid content (41.26%) was obtained in the following condition: pH equal to 7, C/N ratio equal to 70, agitation speed equal to 300 rpm and initial cell density equal to 0.8. Nevertheless, it was not possible to determine in this work the optimal condition for lipid production. In the condition afore mentioned, the fatty acid profile of *P. laurentii* UFV-1 was determined: 0.55% C14:0, 26.31% C16:0, 8.05% C18:0, 0.57% C20:0, 10.57% C16:1, 45.5% C18:1, 0.36% C20:1, 0.18% C18:3 and 17.19% C18:2. This profile is similar those found for soybean oil. Since this edible oil is currently used for biodiesel production, we conclude that lipid extracted from *P. laurentii* UFV-1 are suitable for its production.

RESUMO

DOS SANTOS, Raquel Cristina Vieira, M.Sc., Universidade Federal de Viçosa, agosto de 2017. **Avaliação do efeito das condições de crescimento sobre a produção de lipídeos pela levedura *Papiliotrema laurentii* UFV-1 em meio de cultura contendo xilose.** Orientador: Wendel Batista da Silveira. Coorientador: José Ivo Ribeiro Júnior.

Os biocombustíveis são uma alternativa para reduzir a dependência do uso de combustíveis fósseis. Dentre eles, o biodiesel produzido a partir da reação de transesterificação entre um álcool e uma molécula de triacilglicerol é um substituto ao diesel convencional. Atualmente, os óleos vegetais comestíveis são as principais fontes de triacilgliceróis para a produção de biodiesel. No entanto, a produção desses óleos compete com a produção de alimentos por terras aráveis e água. Além disso, as características dos óleos vegetais são afetadas por condições climáticas. Esses inconvenientes podem ser contornados usando fontes alternativas de óleo, como os óleos de microrganismos. Leveduras oleaginosas acumulam pelo menos 20% da sua massa seca como lipídeos, principalmente na forma de triacilgliceróis, no entanto, valores superiores podem ser alcançados dependendo das condições de cultivo. *Papiliotrema laurentii* UFV-1, ao contrário da levedura oleaginosa modelo *Yarrowia lipolytica*, é capaz de crescer e acumular lipídeos a partir de xilose, o principal açúcar encontrado na hemicelulose. Sendo assim, esse trabalho teve como objetivo avaliar os efeitos do pH, razão C/N, agitação, densidade celular inicial e temperatura sobre a produção de lipídios por *P. laurentii* UFV-1, uma linhagem de levedura isolada recentemente do solo. Nós realizamos um delineamento composto central com face centrada, com 25 combinações entre os fatores estudados e três repetições no ponto central, para avaliar seus efeitos sobre a variável-resposta conteúdo lipídico. O maior conteúdo lipídico (41,26%) foi obtido na seguinte condição: pH igual a 7, razão C/N igual a 70, agitação igual a 300 rpm e densidade inicial de células igual a 0,8. Não foi possível determinar neste trabalho a condição ótima para a produção de lipídios. Na combinação mencionada acima, o perfil de ácidos graxos de *P. laurentii* UFV-1 foi o seguinte: 0,55% C14:0, 26,31%, C16:0, 8,05% C18:0, 0,57% C20:0, 10,57% C16:1, 45,5% C18:1, 0,36% C20:1, 0,18% C18:3 e 17,19% C18:2. Esse perfil é semelhante ao encontrado no óleo de soja. Uma vez que este óleo comestível é

atualmente usado para a produção de biodiesel, concluímos que os lipídios extraídos de *P. laurentii* UFV-1 são adequados para a produção desse biocombustível.

1. Introduction

Most of the energy used in the world is still obtained from the burning of fossil fuels: petroleum, coal and natural gas. However, these sources are distributed heterogeneously along the continents, besides their combustion leads to emission of carbon dioxide (CO₂) into the atmosphere which is associated with the increase in Earth's temperature over the last decades.

Gasoline and diesel oil, which is also known as conventional diesel or mineral diesel, are the major liquid fuels obtained from petroleum refining and are responsible for moving cars and vehicles with cycle-diesel engines, respectively.

With the increase of the population and industrialization, the use of gasoline and diesel represent a considerable source of CO₂ emissions. In this sense, many countries have adopted policies and strategies for the use of biofuels, which are produced from renewable and sustainable feedstocks. Bioethanol is an alternative to gasoline while biodiesel has been used as a substitute for conventional diesel.

Biodiesel displays various advantages over diesel: less polluting, renewable, environmentally friendly, non-toxic and biodegradable. A short chain alcohol and a triacylglycerol molecule are required for its manufacture. Currently, biodiesel production takes place from edible oils, however their use have some drawbacks such as the competition for arable lands and the changes in their composition due to environmental conditions. In this sense, the triacylglycerols extracted from oleaginous microorganisms has been considered a promising oil source for biodiesel production.

These microorganisms can accumulate over 20% of their dry weight as lipid, mainly in the form of triacylglycerols. Depending on the cultivation conditions, which include pH, temperature, agitation speed and the carbon/nitrogen (C/N) ratio, they can reach values close to 75%. The C/N ratio is pivotal for inducing lipid accumulation, because in environments with high carbon concentration relative to nitrogen, oleaginous microorganisms can convert the excess carbon to triacylglycerols, which are stocked in cellular organelles referred as lipid droplets.

Yeasts highlight among the oleaginous microorganisms as being are easily cultivated in bioreactors, besides their fatty acid profile is similar to the found in soybean oil, which is used in the manufacture of biodiesel. The yeast *Papiliotrema laurentii* UFV-1 was isolated from a soil sample of the Serra dos Órgãos Park (Rio de

Janeiro, Brazil, latitude S 22° 27' 33.1" and longitude W 43° 01' 40.0") and selected due to its capacity to accumulate lipids when cultured in media containing xylose as the sole carbon and energy source. So far, the factors that affect the production of lipids by this strain yeast still were not evaluated.

P. laurentii, previously classified as *Cryptococcus laurentii*, is capable growing in several carbon sources such as glucose, xylose, arabinose, lactose, fructose, cellobiose, raffinose, glycerol and polysaccharide starch. The remarkable ability of assimilating several sugars highlights its potential to be used in fermentative processes.

Xylose, a sugar of five carbons, is the most abundant constituent of the hemicellulose of lignocellulosic biomasses, which are the most abundant renewable source in nature. Since the xylose conversion into ethanol by yeasts conventionally used in industrial processes is not efficient, this sugar can be used for production of the other biochemicals and biofuels such as lipids, xylitol, lubricants, ethanol and fatty alcohol.

This work aimed evaluate the lipid production by *P. laurentii* from culture media containing xylose as the sole carbon source. The effects of pH, C/N ratio, agitation speed, initial cell density and temperature on lipid production were evaluated.

2. Aims

2.1 General aim

Evaluate the effect of five factors associated with growth conditions: pH, agitation speed, C/N ratio, temperature and initial cell density on production of lipids by *P. laurentii* UFV-1 in culture media containing xylose as the sole carbon source.

2.2 Specific aims

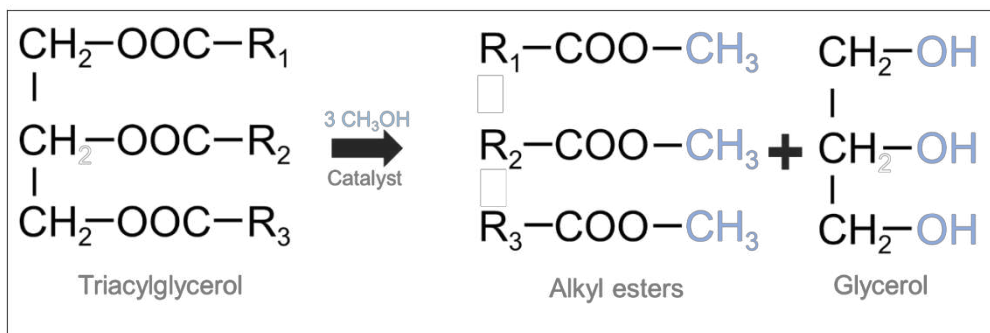
- Identify the factors that affect the production of lipids in *P. laurentii* UFV-1;
- To obtain an empirical model to correlate the factors studied and production of lipids using the response surface methodology (RSM);
- To obtain the levels of factors that increase the production of lipids;

- To determine the profile of fatty acids in the best condition growth condition found and to predict the physicochemical characteristics of biodiesel produced in this situation.

3. Review

Biofuels are an alternative to reduce greenhouse gases such as CO₂. Biodiesel is a biofuel that can be used as an alternative to the diesel produced by fractional distillation of petroleum as it is lesser polluting and aggressive to the environment than the diesel (CASPETA; NIELSEN, 2013; DUARTE et al., 2013; HOEKMAN et al., 2012). In Brazil, since 2008, it became mandatory the addition of 2% of biodiesel to petroleum diesel, and this value is increasing over the years, being expected to reach 15% up to 2019 (PLANALTO, 2016).

The production of biodiesel is performed by transesterification reaction, in which three alcohol molecules react with one triacylglycerol molecule in the presence of a catalyst, which may be acidic, basic or enzymatic. In this reaction is produced a mixture of fatty acid esters, corresponding to biodiesel, and glycerol, as by-product. This reaction is shown in figure 1. When methanol and ethanol are the alcohols used in the transesterification reaction, biodiesel is referred as fatty acid methyl esters (FAME), and fatty acid ethyl esters (FAEE), respectively (HOEKMAN et al., 2012, BUIJS;



SIEWERS; NIELSEN, 2013).

Vegetable oils are commonly used in the production of biodiesel. In Brazil, soybean oil is the main triacylglycerol source, although other plants such as jatropha, canola

and palm also have been tested. Furthermore, animal fats such as beef tallow and fish oil have been considered (GALAFASSI et al., 2012).

Nevertheless, the use of edible oils for biodiesel production has many drawbacks including the competition with arable land and water, which could be used in the cultivation of food (CHEIRSILP; LOUHASAKUL, 2013), and dependence on weather conditions, which affect the productivity of the plant and the physical and chemical characteristics of the oil (TANIMURA et al., 2014 a).

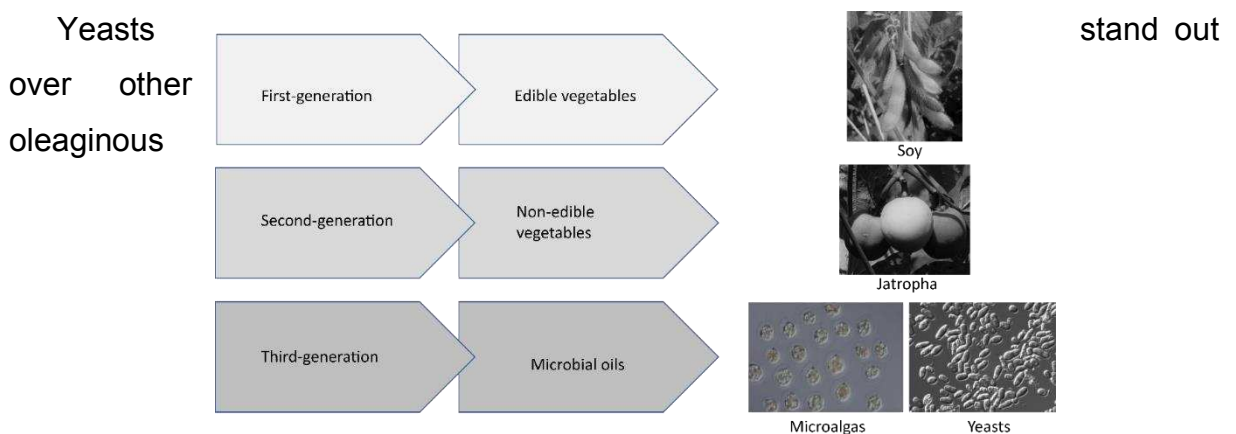
Oleaginous microorganisms are an alternative oil source that have gained attention over the last years. In contrast to the majority of microorganisms that normally produce about 6 to 8% of their dry weight as lipids, oleaginous microorganisms have the ability of accumulating between 20% and 70% of their dry weight as lipids, mainly in the form of triacylglycerols (CALVEY et al., 2016; SCHULZE et al., 2014; SITEPU et al., 2013; TANIMURA et al., 2016). The oil produced by these microorganisms can be extracted by organic solvents and depending on its fatty acid composition can be used for different purposes. In the food industry, they can be used as a polyunsaturated fatty acids (PUFAs) source which present high nutritional value and health benefits. In the cosmetic industry, they have been used for cosmetic production due to its moisturizing and emollient features. The Brazilian company Natura has used oils extracted from microalgae in the composition of brand product (NATURA CAMPUS, 2016). In addition, microbial oil can be used for third-generation biodiesel production.

According to Lee and contributors (LEE; KIM; CHEON, 2015) third generation biodiesel is produced from microbial lipids, while the first generation is produced from edible vegetables such as soybean. Whereas the second-generation biodiesel, non-edible oils are used as feedstocks such jatropha. The third-generation biodiesel does not compete with food production; thus, it is considered a promising process in terms of sustainability. Biodiesel generations are shown in figure 2 below:

Figure 2 Biodiesel generations and its raw materials. Authorial figure, source of images: Pixabay and Wikimedia Commons.

The main challenges to make third generation biodiesel feasible are: selection of appropriate microorganisms, optimization of culture conditions for improving the lipid production, and the development of an efficient process of oil extraction.

Microalgae are also studied for lipid production. However, they require large spaces to be cultured and need to be exposed to controlled amounts of sunlight (AMI et al., 2014). Bacteria are also able to produce lipids, however they are accumulated in their outer membrane, requiring a laborious extraction process (TANIMURA et al., 2014b). In addition they accumulate lipid as polyhydroxyalkanoates (PHA), which are not suitable for production of biodiesel (MENG et al., 2008).



microorganisms, due to following features: availability of tools for genetic manipulation, and ease of growing in bioreactors (PAPANIKOLAOU; AGGELIS, 2011; POLI et al., 2013).

Oleaginous yeasts accumulate lipids in specialized cellular compartments known as lipid bodies or lipid droplets (BEOPOULOS et al., 2009). The species that are most commonly studied include: *Yarrowia lipolytica*, *Rhodotorula glutinis*, *Lipomyces*

starkeyi, *Cryptococcus* and *Rhodospiridium toruloides* (SITEPU et al., 2014 b). In general, these yeasts have been isolated from cheese, yogurt, kefir, marine environments, sewage, soy sauce, meat, soil samples, stems and flowers (GONÇALVES; COLEN; TAKAHASHI, 2014; NICAUD, 2012; TANIMURA et al., 2014b).

Oils synthesized by oleaginous yeast can be used for the production biodiesel without affecting the features of the final product, because their fatty acid profile is similar to that found in plants. The predominant fatty acids are myristic acid (C14: 0), palmitic acid (C16: 0), palmitoleic acid (C16: 1) stearic acid (C18: 0), oleic acid (C18: 1) and linoleic acid (C18: 2). The presence of oleic acid (C18: 1) is highly desirable because it improves the ignition properties of the biodiesel (BEOPOULOS; NICAUD; GAILLARDIN, 2011; SITEPU et al., 2014c). Tanimura and contributors (TANIMURA et al., 2014 b) showed that the biodiesel produced from the oil extracted from yeast displays the same physical-chemical characteristics that the biodiesel manufactured from jatropha; which meets the requirements for the biodiesel market in the United States and European Union.

The lipid accumulation in oleaginous yeasts is related to carbon/ nitrogen (C/N) ratio in the culture medium. In an environment where there is excess of carbon and nitrogen limitation, the biosynthesis of proteins and nucleic acids required for cell proliferation decreases, and excess carbon is funneled for oil production (lipogenesis). In addition to the C/N ratio, considered the main factor for lipid biosynthesis in oleaginous yeasts, factors such as pH, temperature and aeration of the medium are also important as are related to cell growth and may affect some characteristics of the fatty acids that are synthesized by the cell such as the size of the carbon chain and the degree of saturation (CALVEY et al., 2016; GONÇALVES; COLEN; TAKAHASHI, 2014).

There are two routes of carbon accumulation in microorganisms, in the first one the excess is in the form of sugars, for example glucose known as de novo synthesis. In the second one, carbon is accumulated in the form of hydrophobic substrates, such glycerol or vegetable oils, called via accumulation ex novo (BEOPOULOS et al., 2009, GONÇALVES; COLEN; TAKAHASHI, 2014).

The de novo synthesis is favored under nitrogen limitation. In this condition, AMP is cleaved to IMP and NH_4^+ , thereby the isocitrate dehydrogenase (enzyme of the TCA

cycle), which is AMP dependent, does not convert isocitrate to α -ketoglutarate. The excess of isocitrate is then converted to citrate by the aconitase enzyme and then it is transported to the cytoplasm where it will be converted into oxaloacetate and acetyl-CoA by ATP citrate lyase. It should be pointed out that acetyl-CoA is the precursor molecule of the triacylglycerol biosynthesis. The citrate conversion to acetyl-CoA by ATP citrate lyase occurs only in oleaginous yeasts (JIN et al., 2015). The de novo synthesis is shown, in summary, in the figure 3.

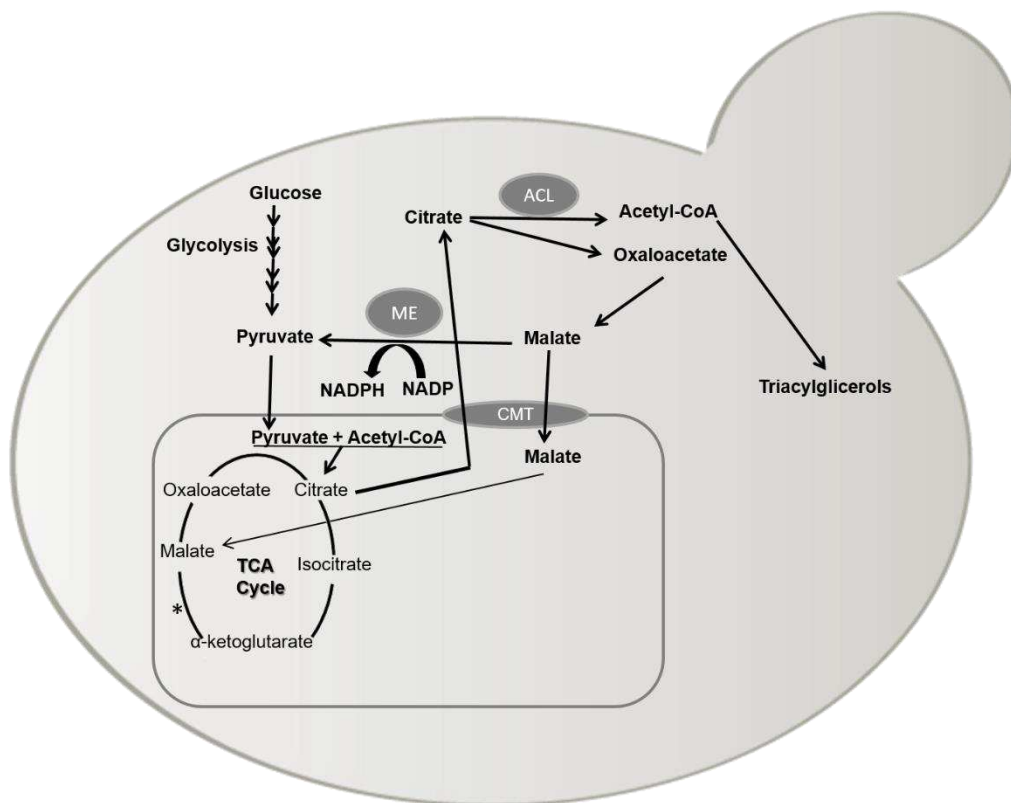


Figure 3 The de novo synthesis of triacylglycerols in oleaginous yeast. ACL: ATP citrate lyase, ME: malic enzyme, CMT: citrate/malate translocase. Authorial figure.

acids are metabolized. Fatty, lipase and phospholipase are released into the extracellular medium in order to hydrolyze those substrates into products that are transported to the interior of cell, where the lipids are reassembled and stored (GONÇALVES; COLEN; TAKAHASHI, 2014).

The *P. laurentii* UFV-1 yeast was isolated by VIEIRA, 2018 on a soil sample of the Brazilian National Park Serra dos Órgãos (Rio de Janeiro, Brazil) and selected due to its ability to accumulate lipids from medium containing xylose. 6 *P. laurentii* species, previously designated as *Cryptococcus laurentii* (LIU et al., 2016), were reclassified.

In contrast to *Yarrowia lipolytica*, oleaginous yeast model, *P. laurentii* is capable of converting different sugars such as D-xylose, L-arabinose, cellobiose, fructose and rhamnose into triacylglycerols (CASTANHA et al., 2014; POLBUREE; YONGMANITCHAI; STADLER, 2015; SITEPU et al., 2014).

Xylose is the major pentose found in the hemicelluloses, an important fraction of lignocellulosic biomasses, which are abundant feedstock, as show in figure 4, elaborated with data from HORN et al., 2012. The biorefinery concept of lignocellulosic biomass is directly linked to the conversion of all fractions in some kind of value-added product (DONG et al., 2016; HUANG et al., 2012; PATEL et al., 2016). The glucose released via cellulose hydrolysis is used for second-generation ethanol, however, the conversion of xylose into ethanol is not feasible. Therefore, a promising alternative would be the use of xylose as substrate for triacylglycerols production by oleaginous yeast capable of using xylose as the sole carbon and energy source.

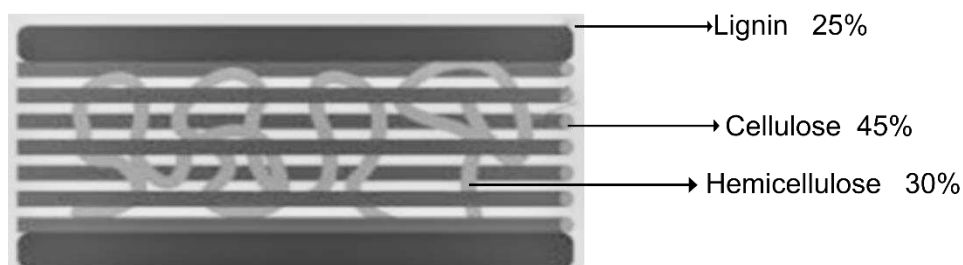


Figure 4 Composition of lignocellulosic biomass. Authorial figure, source of images: Wikimedia Commons.

4. Materials and Methods

4.1 Yeast strain

The microorganism used in this work was the *Papiliotrema laurentii* UFV-1 yeast belonging to the Collection of Cultures of Oleaginous Yeasts of the Microbial Physiology Laboratory at Federal University of Viçosa. This yeast was isolated from soil samples collected in Brazilian National Park Serra dos Órgãos (S 22° 27' 33.1", W

43° 01' 40") and selected due to its ability of growing and accumulating lipids from xylose as the sole carbon source.

4.2 Maintenance of culture

The *P. laurentii* UFV-1 yeast was inoculated into YP medium 10 g/L yeast extract (Himedia), 20 g/L peptone bacteriological (Himedia) with glycerol 100% (Vetec) and stored frozen at – 80 °C.

4.3 Growth conditions

P. laurentii UFV-1 was precultured overnight in YPX medium 10 g/L yeast extract (Himedia), 20 g/L peptone bacteriological (Himedia Laboratories, Vadhani Industrial Estate, Lal Bahadurbai, India)) and 20 g/L xylose (Sigma Chemical Co., MO, USA) at 30 °C, stirring at 200 rpm. To evaluate the lipid accumulation, the yeast was cultured in minimal media composed of ammonium sulfate (concentrations ranging 1.08 g/L from to 1.90 g/L), 0.5 g/L magnesium sulfate hydrated, 0.1 g/L sodium chloride, 0.1 g/L calcium chloride and 0.1 g/L yeast extract and xylose 40 g/L. The yeast cultures were grown in 500 mL Erlenmeyer flasks filled to one-fifth of their capacity with the medium aforementioned. These were incubated in an orbital shaker NEW BRUNSWICK SCIENTIFIC 25D at 30 °C, 32.5 °C and 35 °C, with agitation speed shown in table 1. The citrate-phosphate buffer, 30 mM was used to set pH according to the values established (5, 6 or 7, see table 1).

The cell density was measured spectrophotometrically at 600 nm (DO 600 nm) and cell mass (CDW) gravimetrically.

4.4 Experimental design

4.4.1 Factorial

A factorial design $2^5 + 1$ was applied to determine both the importance and effect of the five independent factors listed in table 1 on the dependent variable, content lipid (%). The experiment consisted of 32 experimental units corresponding combinations between the coded levels of the variables, -1 and +1, plus the central point (0), with

three repetitions, totaling 35 assays, shown in table 2, the experiment was conducted by completely randomized design.

Table 1 Levels of the independent variables according to the factorial design $2^5 + 1$.

Controllable factor	Codel Level		
	-1	0	+1
Agitation speed (rpm)	150	225	300
Biomass inicial (DO _{600nm})	0.2	0.5	0.8
C/N ratio	40:1	55:1	70:1
pH	5	6	7
Temperature (°C)	30	32.5	35

Table 2 Treatments factorial design $2^5 + 1$ conducted to determine variables significant on lipid content (%) and lipid yield (g/L) in *P. laurentii* UFV-1 (the table shows experimental nonrandomized).

Treatment	C/N ratio	Agitation	pH	Biomass cell inicial	Temperature
1	40:1	150	5	0.2	30
2	70:1	150	5	0.2	30
3	40:1	150	5	0.2	35
4	70:1	150	5	0.2	35
5	40:1	300	5	0.2	30
6	70:1	300	5	0.2	30
7	40:1	300	5	0.2	35
8	70:1	300	5	0.2	35
9	40:1	150	7	0.2	30
10	70:1	150	7	0.2	30
11	40:1	150	7	0.2	35
12	70:1	150	7	0.2	35
13	40:1	300	7	0.2	30

14	70:1	300	7	0.2	30
15	40:1	300	7	0.2	35
16	70:1	300	7	0.2	35
17	40:1	150	5	0.8	30
18	70:1	150	5	0.8	30
19	40:1	150	5	0.8	35
20	70:1	150	5	0.8	35
21	40:1	300	5	0.8	30
22	70:1	300	5	0.8	30
23	40:1	300	5	0.8	35
24	70:1	300	5	0.8	35
25	40:1	150	7	0.8	30
26	70:1	150	7	0.8	30
27	40:1	150	7	0.8	35
28	70:1	150	7	0.8	35
29	40:1	300	7	0.8	30
30	70:1	300	7	0.8	30
31	40:1	300	7	0.8	35
32	70:1	300	7	0.8	35
33	55:1	225	6	0.5	32.5

4.4.2 Construction and analysis of the response surface

The response surface methodology (RSM) was used for statistical modeling and optimization of response factor lipid content (%). The Minitab software, version 16 was used to generate the mathematical model that describes the relation between the four independent variables and the response factor lipid content, and the variance analysis (ANOVA) of the adjusted model. The full quadratic model was analyzed, including linear effects, double interactions and quadratic interactions between the independent

variables. According to the t-test ($\alpha=0.05$), the terms nonsignificant statistically were eliminated wherever possible, for simplification of the model.

From the model provided by Minitab software version 16, the predicted response surfaces were generated using Sigma Plot software version 12. We analyzed the effect of two factors under the response, at one time setting the others in the value that maximized the lipid content.

4.5 Extraction and quantification of lipids

After 72h of growth the cells were harvested by centrifugation and lyophilized for 24h. Approximately 50 mg of the obtained material was used for the extraction and quantification of the lipid content. The lipid extraction was performed according to the methodology proposed by Bligh and Dyer (1959), employing methanol and chloroform (2:1), but saline solution (NaCl 1%) was used in the last step of the procedure in order to improve the separation between the aqueous phase and the organic phase.

4.6 Fatty acid profile analysis

The fatty acid profile was analyzed by a gas chromatograph with flame ionization detector, GC-FID 6890N, Agilent Technologies 7890A ®. Samples from *P. laurentii* UFV-1 were harvested by centrifugation at 12000 $\times g$ at 4 °C for 10 min, and the pellet was lyophilized. The fatty acids in the yeast cells (1 mg dry weight) were saponified, methylated, extracted and identified using the MIDI microbial identification system (Sherlock 6.0 microbial identification system) at Microbial ID, Inc. (Newark, DE).

4.7 Prediction of fatty acid quality for biodiesel production

From fatty acid profile obtained in item 3.6, the following biodiesel properties were estimated: iodine value (dimensionless), cetane number (dimensionless), cloud point (°C) higher heating value (MJ/kg) kinematic viscosity (mm^2/s) and density (g/cm^3) according Klopfenstein, W.E., 1985, Krisnangkura, K., 1986 and Biodiesel Analyzer © software version 2.2, Online App (<http://www.brteam.ir/biodieselanalyzer>).

4.8 Consumption of xylose and ammonium sulfate

The xylose consumption was quantified by high performance liquid chromatography (HPLC), in a chromatograph Shimadzu TA-20, using a Aminex column (HPX-87H 300×7.8 mm, 9 μm, Bio-Rad, Munich, Germany) 0.5 mM H₂SO₄ as the mobile phase, an injection volume of 10 μL, and refractive index detector (RID-20A, Shimadzu, Japan). Xylose quantification was obtained by calibration curves using external standards

The ammonium sulfate consumption was measured following the colorimetric method proposed by Chaney and Marbach (1962), based on the reaction of Berthelot. A calibration curve was constructed with different ammonium sulfate concentrations (20- 200 mg/L).

4.9 Determination of fermentation parameters

The following parameters were calculated: cell mass production (g cell dry per liter culture), lipid content (g lipid per g cell dry) lipid total (g lipid per liter of culture), and lipid productivity (mass lipid per volume culture per time).

5. Results and Discussion

We determined the lipid content [% (w/w)] after 72 h of growth for all cultivations, which corresponded to 35 combinations between the variables shown in table 2. *P. laurentii* UFV-1 did not grow well at 35 °C; thus, the quantity of biomass required to extract lipids was not achieved. For this reason, temperature was not included as variable and the factorial design was altered. All cultivations were carried out at 30 °C and the factorial design focused on evaluating the effects and interactions between the variables e C/N ratio, agitation, pH and initial biomass on the response variable. Thus, 8 new combinations between the central point and the levels, codified as -1 and +1, of each variable were included, generating a centered face central composite design. This new design, consisted of 24 experimental units corresponding to combinations of the encoded levels -1 and +1 plus central point, with three repetitions totaling 27 assays. The experiment was conducted by completely randomized design.

The factor levels and their operating regions are shown in table 1. It was evaluated whether there were synergistic or antagonistic relationships between the factors aforementioned. Each treatment was evaluated by the response surface methodology (RSM) and quadratic polynomial model obtained is shown by equation 1.

$$\text{Lipid content (\%)} = 0.2609^* + 0.02135^* \text{ C/N ratio} + 0.00686^* \text{ Agitation speed} + 0.02283^* \text{ pH} + 0.01682^* \text{ Biomass} + 0.0379^* (\text{Agitation})^2 + 0.0413^* (\text{Biomass})^2 + 0.01782^* (\text{Agitation}) (\text{Biomass}), R^2 = 0,75 \text{ (Equation 1)}$$

* = significant T-test, (p-value <0.05).

Table 3 Regression analyses for lipid content (%)

Term	Effect	Coef	T-value	p-value
Constant		0.2609	22.55	0.000
C/N rate	0.04270	0.02135	2.78	0.012
Agitation	0.01372	0.00686	0.89	0.383
pH	0.04567	0.02283	2.97	0.008
Biomass	0.03363	0.01682	2.19	0.041
Agitation*Agitation speed	0.07580	0.03790	2.12	0.047
Biomass*Biomass	0.08260	0.04130	2.31	0.032
Agitation*Biomass	0.03564	0.01782	2.19	0.042

Coef: adjusted coefficient; **T-value:** t-test value; **p-value:** p-value

The terms statistically no significant of the equation were eliminated to simplify the model, which correspond to the double interactions, except the interaction between agitation speed and biomass, and all squared terms, except (Biomass)² and (Agitation)². The F test (8.06) of the analysis of variance and p-value (0.000) indicate that the model obtained is significant. The low correlation coefficient R² obtained (0.7482) is explained by the fact that two square terms (C/N ratio and pH) were not

incorporated into the model. However, it should be pointed out that there was a correspondence between the values obtained experimentally and the predicted values.

All coefficients of the linear terms, i.e, C/N ratio, pH, agitation speed and initial biomass were significant (p-value <0.05) and affect the response factor lipid content. Since their coefficients are linear and positive, the increase in the level of them, compared to the levels evaluated in this work, suggests the increase of the response factor.

The coefficients C/N ratio and pH presented higher magnitude; thus, they are the most important effects regarding the accumulation of lipids by the yeast *P. laurentii* UFV-1. Therefore, they should be considered in future studies in order to find the levels that maximize the lipid production. The model indicated that pH values higher than 7 maximize the response variable; nevertheless, the *P. laurentii* UFV-1 yeast did not grow after 72 h in pH values 9 and 10. Therefore, the pH range studied covered the optimal region and the factor that needs to be evaluated in more detail is the C/N ratio. Regarding the C/N ratio, the model indicated that values higher than the maximum tested, that is, C/N ratio equal to 70, favor the increase of the lipid content.

Indeed, it is well established that the C/N ratio is the most important parameter that affects to lipid accumulation by oleaginous microorganisms (SITEPU et al., 2014, GALAFASSI et al., 2012). Under nitrogen-limiting conditions, the growth rate decreases and the synthesis of proteins and nucleic acids also is reduced; thus, the excess carbon in the medium is channeled into the lipid synthesis, which is not observed in non-oleaginous species.

It is well known that the imbalance between carbon and nitrogen in the culture medium is pivotal for lipid accumulation in oleaginous yeasts, however, the proportion of this imbalance is strain-dependent. Besides, both carbon and nitrogen sources also affect the lipid accumulation. Indeed, *Lipomyces starkeyi* accumulated 18.74% (g/g) and 30.00% (g/g) of lipids in culture media with C/N ratios equal to 24:1 and 48:1, respectively (CALVEY et al., 2016).

Wang et al, 2017 evaluated the lipid production by *P. laurentii* AM 113 from inulin hydrolysate, a polysaccharide constituted of glucose and fructose. Nevertheless, these authors did not observe differences with regard to lipid accumulation from two different C/N ratios, standing out that the C/N ratio effect on lipid accumulation is strain-dependent.

With regard to the effects of both agitation speed and initial biomass, we observed that the coefficients of the square terms and of interaction were significant (p-value <0.05) (table 3). The positive sign of the interaction coefficient between agitation speed and biomass (0.01782), provided by equation 1, shows that the two variables need to increase simultaneously to maximize the lipid content (response variable), that is, as the initial amount of inoculum is increased, higher agitation speed values are required. The maximum agitation speed used in the 25 treatments was 300 rpm for an initial optical density (OD 600 nm) of cells equal to 0.8. Under these conditions, the maximum value of lipid content was not found. Therefore, further experiments should be conducted with agitations superior to 300 rpm. It is noteworthy that the agitation speed is related to the oxygen availability in the culture medium. According to Liu et al. (2008) the amount of oxygen dissolved in the medium may be associated with the amount of accumulated lipids. For example, the *Rhodotorula glutinis* yeast increased its lipid accumulation by 80% in high oxygen level (NIGAM et al., 1999, PAN et al., 1986). On the other hand, *Cryptococcus curvatus*, previously called *Apiotrychum curvatum*, increased its lipid production in low oxygen levels (BOULTON and RATLEDGE 1984)

In the present work, the highest lipid contents (41.26% and 40.35%) were achieved by *P. laurentii* UFV-1 at agitation speed of 300 rpm. However, it should be pointed out that even at agitation speed of 150 rpm, the lipid contents, 38.14% (treatment 12) and 36.22% (treatment 4), were close to those obtained at 300 rpm. In addition, it was observed in the treatments 16 and 14, in which higher agitation speeds were adopted, that nitrogen was depleted faster than in other conditions (Figure 6). Thus, we believe that the higher agitation speed favored the nitrogen consumption, which in turn, altered the C/N ratio leading to a greater lipid accumulation. Indeed, in the treatments 4 and 12, which were conducted at low agitation speed, the nitrogen depletion occurred only after 48 hours of growth (Figure 6), i.e, slower than in the treatments 16 and 14. Even though lipid contents have been lower in treatments 4 and 12 compared to 16 and 14, their values are among the highest obtained in the 25 treatments. Similarly, Calvey et al., 2016 observed that the yeast *Lipomyces starkey* cultivated in a culture medium with C/N ratio equal to 72 and agitation speed of 150 rpm accumulated a highest lipid content 44.6% [% (w/w)]. These authors proposed that there is a synergistic effect between low agitation and C/N ratio on lipid accumulation.

Under oxygen limitation, the ATP synthesis drops and there is an excess of NADH. Based on that, the authors claimed that the NADH imbalance is solved via its reduction to NAD⁺ in the fatty acid synthesis pathway, favoring the lipid accumulation. It is important to point out that in the treatments 4 and 12 the low agitation speed is associated with the higher limitation of nitrogen evaluated, this is, with a C/N ratio equal to 70, indicating that both oxygen and nitrogen limitations favored the accumulation of lipids by *P. laurentii* UFV-1.

Table 4 Cell mass production, lipid content, lipid total and lipid volumetric productivity for all treatments studied after 72 hours of culture

Treatment	Cell mass production (g/L)	Lipid content % (g/g)	Lipid total (g/L)	Lipid volumetric Productivity (g/L.h)
1	7.75	25.5	1.98	0.027
2	6.82	33.21	2.26	0.031
3	6.22	37.12	2.31	0.032
4	8.73	36.22	3.16	0.044
5	11.37	27.06	3.08	0.043
6	9.49	30.36	2.88	0.040
7	4.87	32.1	1.56	0.022
8	7.03	34.03	2.39	0.033
9	8.14	29.72	2.42	0.034
10	6.50	32	2.08	0.029
11	7.08	33.52	2.37	0.033
12	6.42	38.14	2.45	0.034
13	4.87	35.24	1.72	0.024
14	19.42	40.35	7.83	0.109
15	21.58	36.54	7.88	0.110
16	26.83	41.26	11.07	0.154
17	15.65	23.47	3.67	0.051
18	27.18	33.13	9.00	0.125
19	10.73	27.77	2.98	0.041

20	27.95	33.38	9.33	0.130
21	9.60	30.4	2.92	0.041
22	15.87	31.24	4.96	0.069
23	11.31	31.61	3.57	0.050
24	15.74	30.71	4.83	0.067
25	13.45	20.87	2.81	0.039
26	14.21	21.63	3.07	0.043
27	12.62	20.53	2.59	0.036

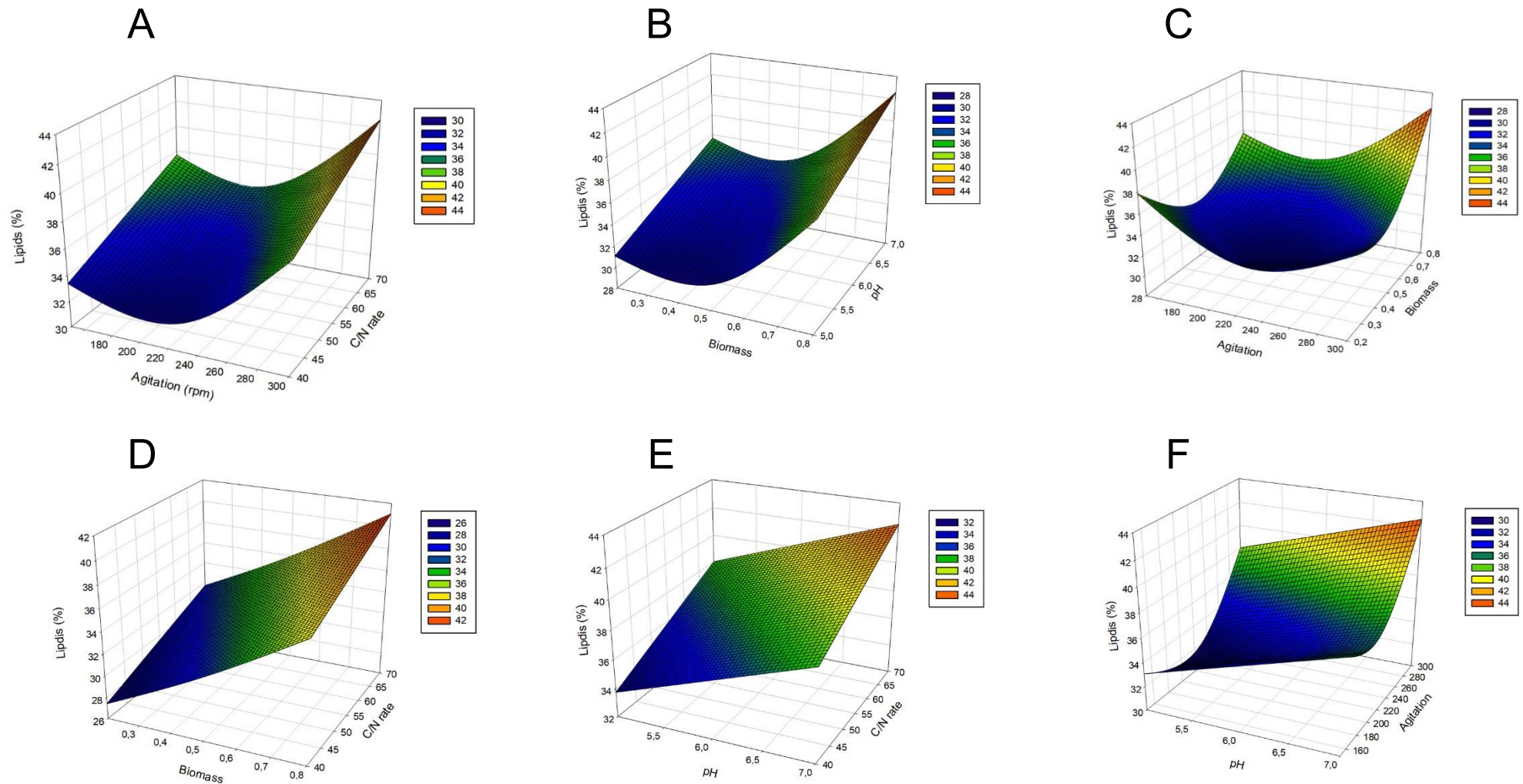


Figure 5 Response surface plot for lipid content (%) as a function of (A) Agitation speed and C/N ratio (B) Biomass and pH (C) Agitation speed and pH (D) Biomass and C/N ratio (E) pH and C/N ratio (F) pH and agitation. For the analysis of the influence of factors two to two under lipid content response the other factors were fixed at their higher coded levels (C/N ratio=1, Biomass=1, Agitation speed=1 and pH=1)

The response surfaces predicted by the model (equation 1) were plotted. The influence of each factor on the lipid content was analyzed by fixing two factors at the levels that displayed the highest response and ranging the other two according to the experimental conditions. In each response surface, the color gradient corresponds to the variation of the lipid content response, with the blue responses being the lowest and the red the highest.

For the factors that displayed only linear terms, i.e., pH and C/N ratio, the surface is fully plan (figure 5E), indicating that the increase in the response is related to the increase of these two factors. The combination between biomass and agitation speed exhibited slight curvatures in the axes as their coefficients have square terms.

The combinations evaluated indicate that it is necessary to work with the maximum level of each factor studied to reach maximum lipid contents. Nevertheless, it should be noted that low agitation speed combined with high C/N ratio also led to the improvement of lipid accumulation (figure 5A), indicating that there was a synergic effect of low agitation and nitrogen limitation.

The surface responses display concavity facing down, showing that the model does not have a quadratic term with a negative and statistically significant signal. None predicted surface represents an optimized condition for lipid production. These results indicate that to achieve values higher than those observed in this work, further experiments or displacements should be performed including other intervals with values higher than those evaluated so far.

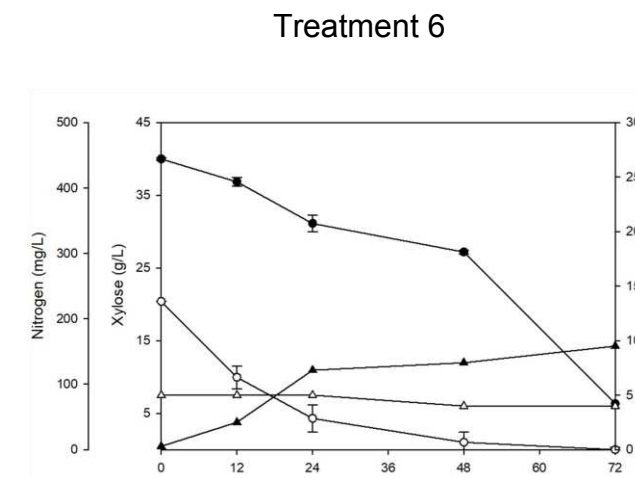
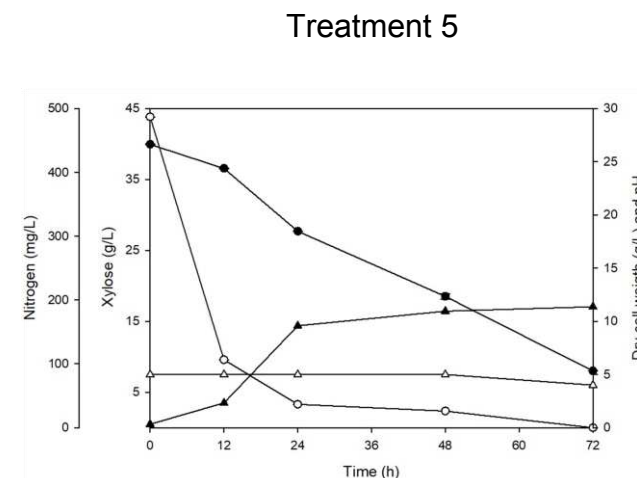
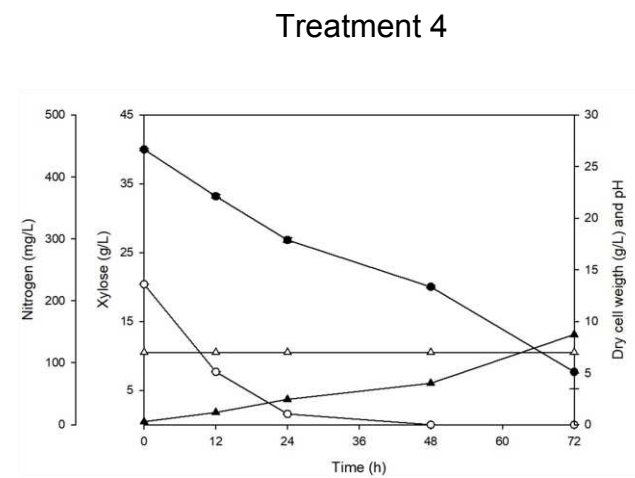
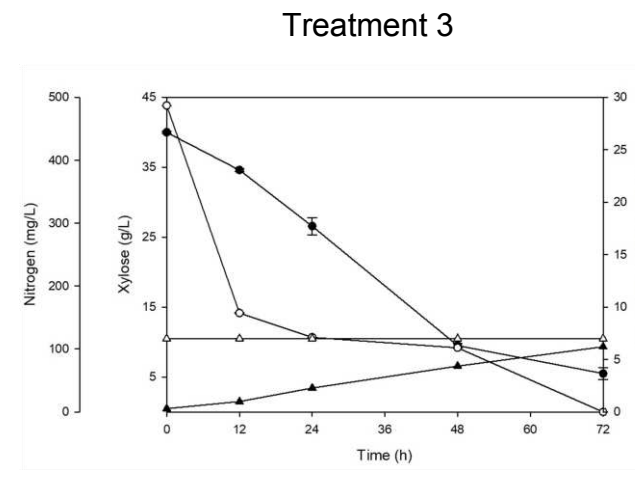
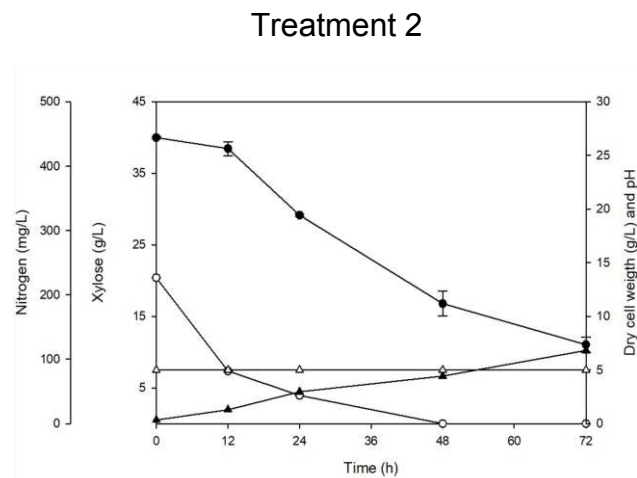
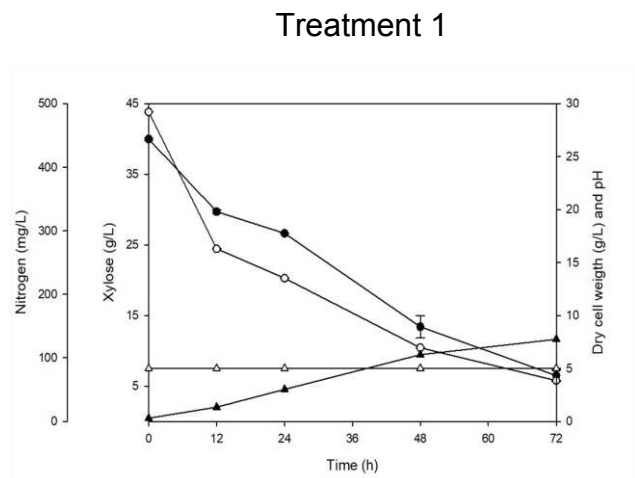


Figure 6a Xylose consumption (●), ammonical nitrogen consumption (○), cell growth (▲) and pH variation (△) over time for each treatment.

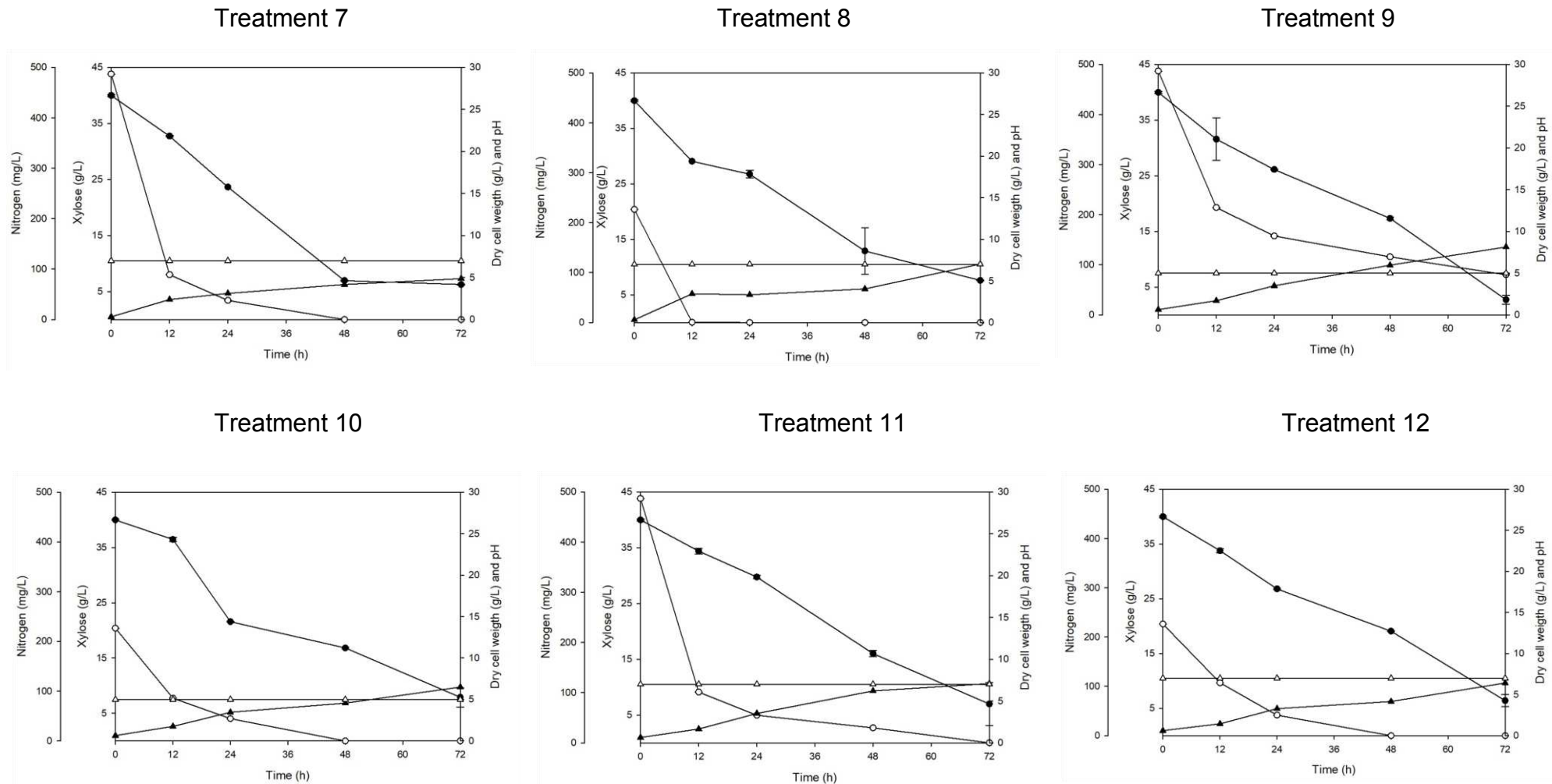


Figure 6b Xylose consumption (●), ammonical nitrogen consumption (○), cell growth (△) and pH variation (▲) over time for each treatment.

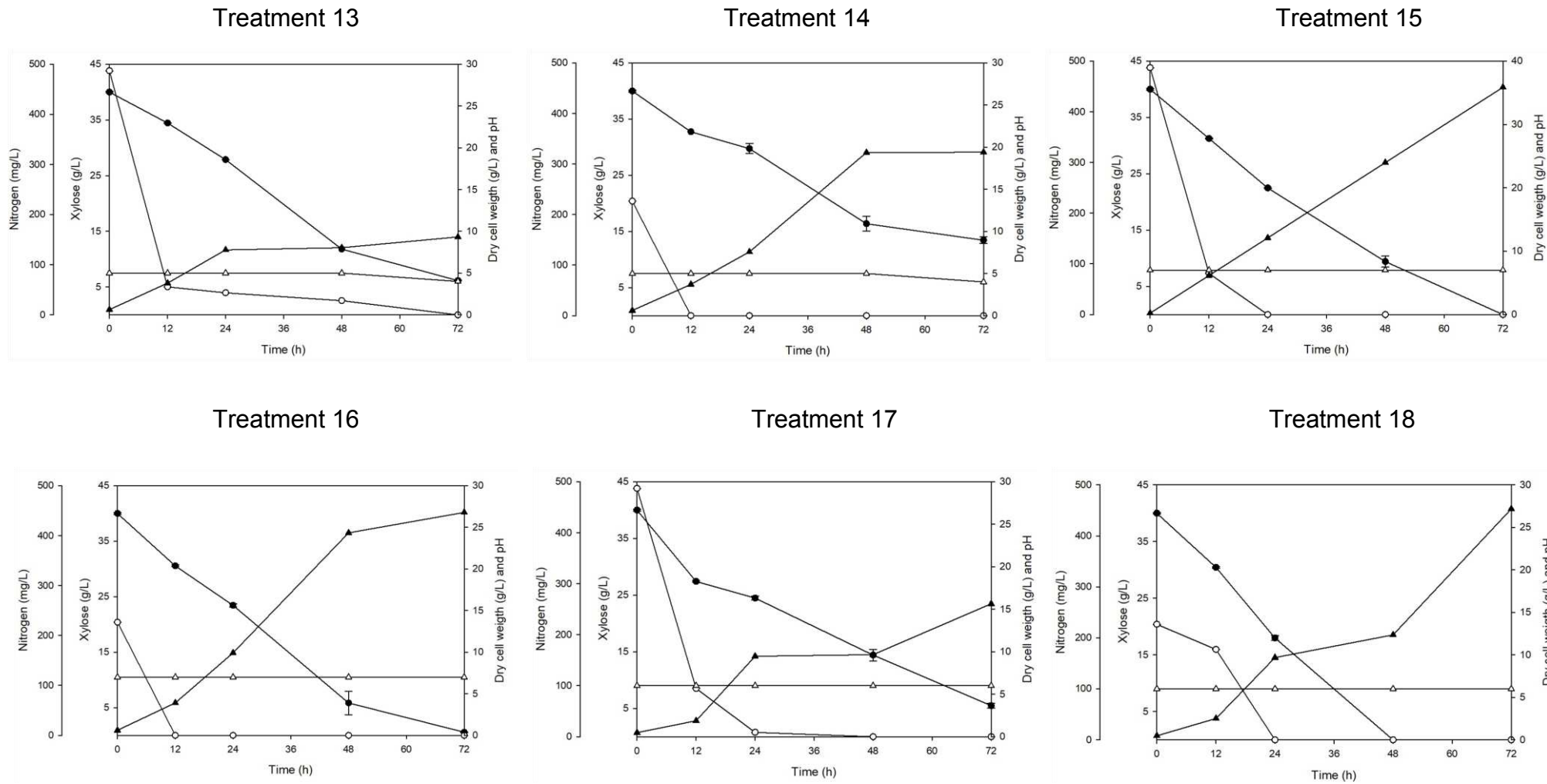


Figure 6c Xylose consumption (○), ammonical nitrogen consumption (●), cell growth (▲) and pH variation (△) over time for each treatment.

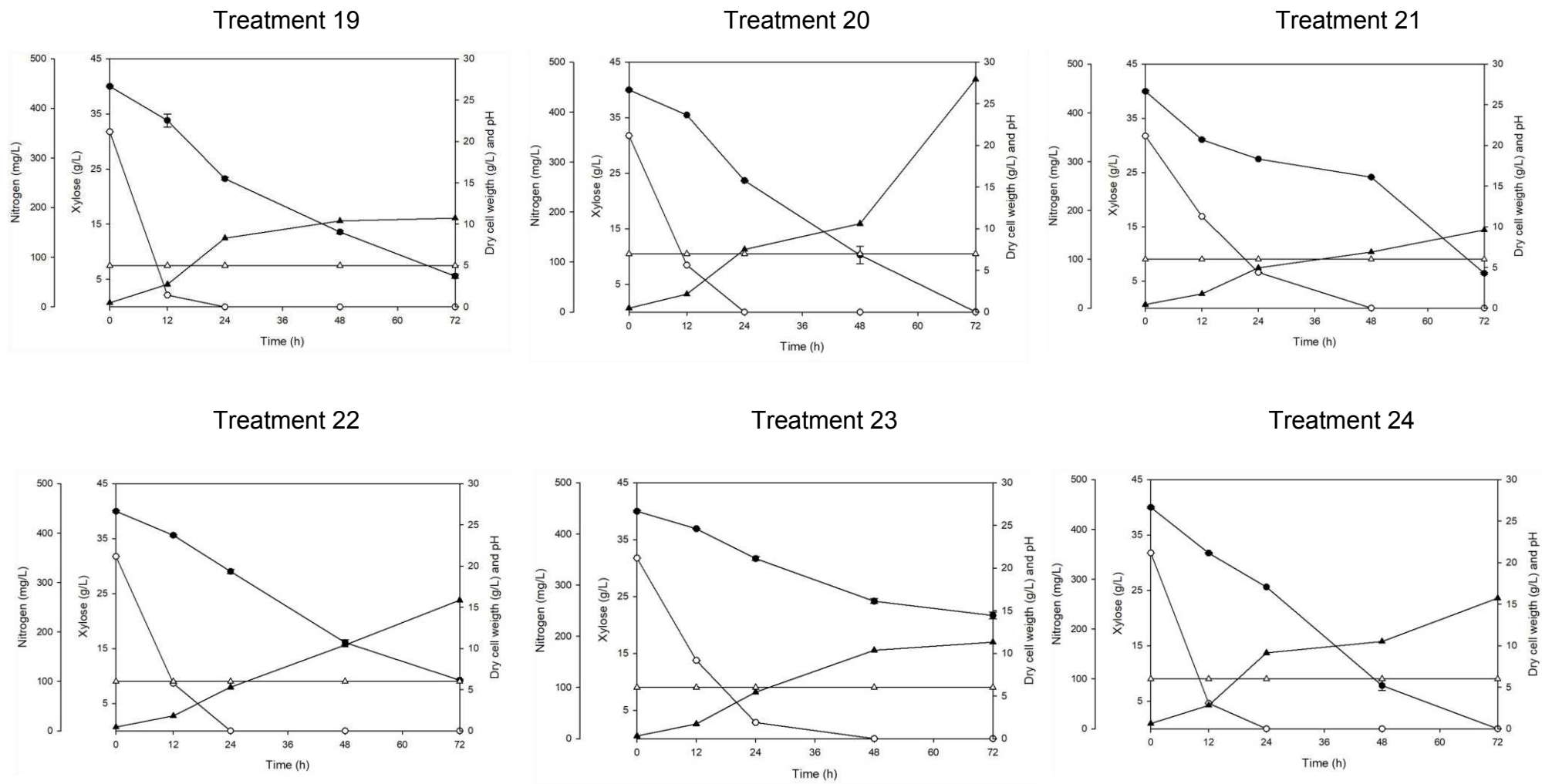


Figure 6d Xylose consumption (○), ammonical nitrogen consumption (●), cell growth (▲) and pH variation (△) over time for each treatment.

Treatment 25

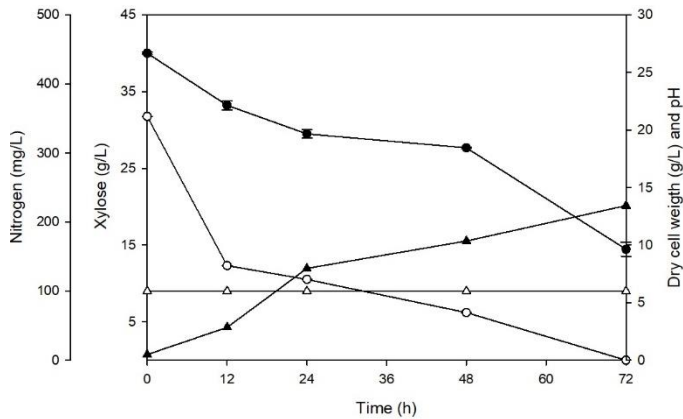


Figure 6e Xylose consumption (●), ammonical nitrogen consumption (○), cell growth (▲) and pH variation (△) over time for each treatment.

The equation 2, which takes into account the lipids contents obtained experimentally to express a quadratic polynomial model, was used in the response surface methodology.

$$\text{Lipid content (\%)} = 0,542 + 0,001423 \text{ C/N rate} - 0,00334 \text{ Agitation} + 0,02283 \text{ pH} - 0,581 \text{ Biomass} + 0,000007 (\text{Agitation})^2 + 0,459 (\text{Biomass})^2 + 0,000792 (\text{Agitation}) (\text{Biomass}), R^2 = 0,75 \text{ (Equation 2)}$$

Equation 2 is useful to estimate the lipid content with different combinations of the variables evaluated, while the equation 1 provides information about the importance of variables tested.

Despite an optimized condition has not been found in this study, the lipid contents achieved were superior to those found (30% w/w) by VIEIRA, 2018 for *P. laurentii* UFV – 1 in medium containing xylose as the sole carbon source.

Taking into account the biodiesel quality, it is desirable that both palmitic and oleic acids are the most abundant fatty acids. Therefore, we evaluated the fatty acid profile of *P. laurentii* UFV-1 in the growth condition that favored the obtainment of the highest lipid content. This condition corresponds to the treatment 16 (C/N ratio 70, pH 7, agitation speed 300 rpm and initial biomass 0.8).

The fatty acid profile showed different fatty acids in the lipid samples (table 5). However, the predominance of C16 and C18 makes the oil stored by *P. laurentii* UFV-1 similar to those found in other oleaginous yeasts and plants such as soybean and palm (table 6). In addition, palm oil is used as feed and raw material for a wide range of products, such as food, plastics, detergents and lubricants (WHIFFI, 2016). Taking into account that soybean and palm are widely used as oil source in the biodiesel production, we concluded that the oil extracted from the yeast aforementioned is also suitable for its production. It is noteworthy that most of the palm oil supplying the world market comes from the extractive exploration of regions of Indonesia and Malaysia, therefore, its use is highly criticized. In this context, the results obtained in the present work highlight the potential of *P. laurentii* UFV-1 as an alternative for third generation biodiesel production, since its oil meets all specifications and can be compared to biodiesel made from soybean and jatropha, which are marketed in several countries. Whiffi et al., 2016 also reported the possibility of using another *P. laurentii* strain as an alternative to replace palm oil.

Table 5 Fatty acid profile of *P. laurentii* UFV-1, other yeasts, soybean and palm oil.

	Fatty acids (%)											Source of carbon	Reference
	14:0 Myristic acid	16:0 Palmitic acid	17:0 Margaric acid	18:0 Stearic acid	20:0 Arachidonic acid	16:1 Palmitoleic acid	18:1 Oleic acid	20:1 Eicosenoic acid	18:3 Linolenic acid	18:2 Linoleic acid	Others		
<i>P. laurentii</i> (treatment 16)	0.55±0.03	26.31±0.05	0±0.00	8.05±0.80	0.57±0.13	0.57±0.18	45.5±0.62	0.36±0.16	0.18±0.26	17.91±1.77	nd	xylose	This work
<i>Pseudozyma hubeiensis</i>	1.3	22.8	nr	16.4	nr	0.5	26.7	nr	nr	21.9	10.5 *	xylose	Tanimura, 2016
<i>Y. lipolytica</i>	nr	11	nr	1	nr	6	28	nr	1	51	nr	glucose	Beopoulos, 2009
Soybean	<0.5	7-11	nr	2-6	<1	nr	19-34	nr	5-11	43-56	23**	-	Ramos, 2009
Palm oil	0.5-2	32-45	nr	2-7	nr	nr	38-52	nr	nr	5-11	nr	-	Ramos, 2009

* 0.2% C12:0, 3.4% C22:0 and 6.9% C24:0

** 23% C22:1

nd: not detected

nr: not reported

Table 6 Comparisons of biodiesel properties from *P. laurentii* UFV-1 with jatropha oil and the US biodiesel standards, EU biodiesel standards and Brazil biodiesel standards.

	Iodine Value	Cetane Number	Cloud Point (°C)	Higher Heating Value MJ/kg	Kinematic Viscosity (mm²/s)	Density (g/cm³)
<i>P. laurentii</i> UFV-1	74.144	56.546	8.847	39.261	3.902	0.87
<i>Jatropha</i> oil (*)	98.02	55.23	4.67	40.55	4.48	nr
Brazil-ANP 255/2003	-	min 45	-	-	-	-
US-ASTM D6751	-	min 47	-	-	1.9-6	-
EU -EN 14214	max 120	min 51	-	-	3.5-5	0.86-0.9

(*) Values reported by Hoekman et al., 2012

Max: maximum

Min: minimum

6. Conclusions

This study concluded:

- *P. laurentii* UFV-1 was capable of accumulating lipids in culture media containing xylose as the sole carbon source, opening perspectives of the application of hemicellulosic fractions as feedstock for lipid production by this yeast;
- In culture media containing xylose as the sole carbon source, the condition in which the yeast had the highest lipid content (41.26%) was the combination of the following factors : C/N ratio 70, agitation speed 300 rpm, pH 7, initial biomass 0.8 (OD_{600nm}) and temperature 30 °C;
- Optimized conditions were not found in this work, and the model obtained for the relationship between the evaluated variables and lipid content indicates that highest values can be achieved by shifting the intervals of the studied variables. In addition, we observed that high lipid contents were reached combining low agitation speed and high C/N ratio, despite these contents have been lower than that obtained in the combination of factors afore mentioned;
- C18:1 (45.5%) and C16:0 (26.31%) are the most abundant fatty acids in the fatty acid profile of *P. laurentii* UFV-1. This profile is similar to that of other oleaginous yeasts and vegetable used in biodiesel manufacture.
- Physicochemical properties predicted for biodiesel made from the oil extracted from *P. laurentii* UFV-1 meet the specifications of Brazilian (ANP 255/2003), American (ASTM D6751) and European (EN 14214) legislation.

7. References

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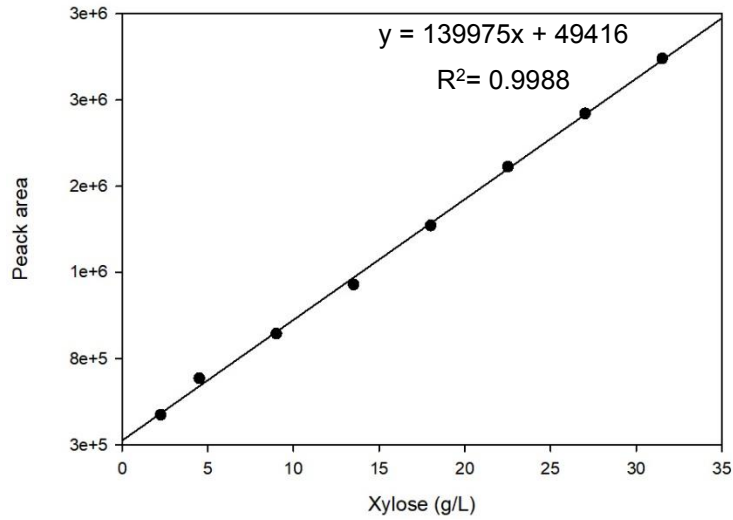
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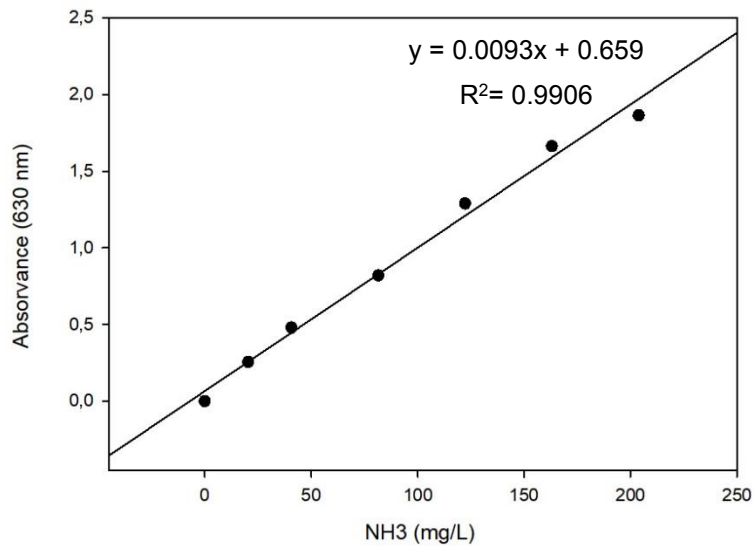
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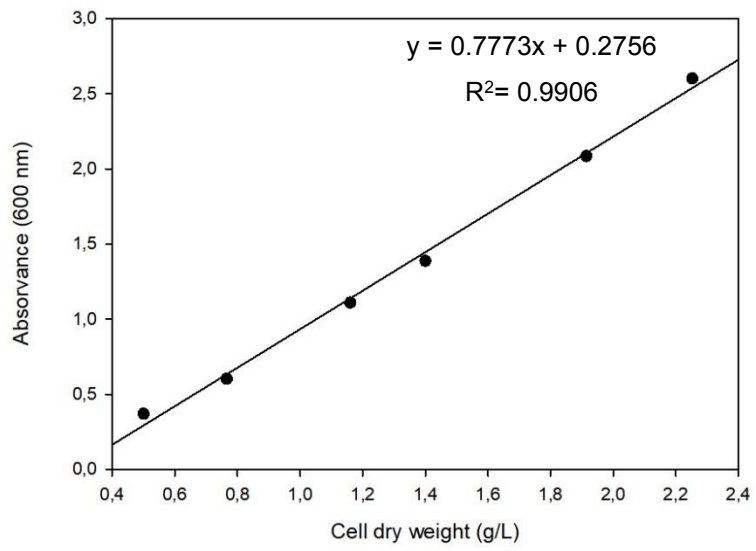
8. Supplementary material



Supplementary figure 1 standard curve of xylose quantification by high performance liquid chromatography (HPLC)



Supplementary figure 2 standard curve of nitrogen ammoniacal quantification by reaction of Berthelot



Supplementary figure 3 Standard curve of dry cell weight of *P. laurentii* UFV-1