FEDERAL UNIVERSITY OF PARANA

LUIZ GUSTAVO LACERDA

DEVELOPMENT OF BIOPROCESS FOR THE PRODUCTION OF MICROBIAL AND ALGAE LIPIDS FROM STARCH TUBERS

CURITIBA

2010

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LUIZ GUSTAVO LACERDA

DEVELOPMENT OF BIOPROCESS FOR THE PRODUCTION OF MICROBIAL AND ALGAE LIPIDS FROM STARCH TUBERS

Thesis presented as partial requirement for obtaining the Doctor degree in Biotechnological Processes, Agroindustry and Biofuels Area. Post-graduation Program in Biotechnological Processes of Technological Sector (Federal University of Parana).

Advisor:

Carlos Ricardo Soccol, Ph.D.

Co-Advisor:

Marco Aurélio da Silva Carvalho Filho, Ph.D.

CURITIBA

2010

RELATÓRIO DE DEFESA DE TESE DE DOUTORADO

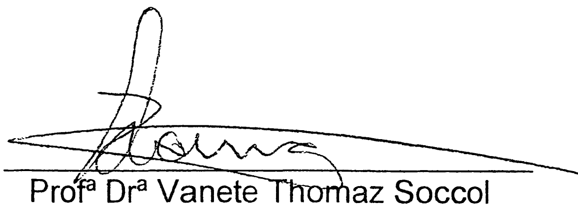
Aos vinte oito dias do mês de dezembro de 2010, na sala da Pós-Graduação em Processos Biotecnológicos, Usinas Piloto, Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pela Profª Drª Luciana Porto de Souza Vandenberghe, Coordenadora do Curso de Pós-Graduação em Processos Biotecnológicos, a banca examinadora para a Septuagésima Defesa de Tese de Doutorado, Área de Concentração: Agroindústria e Biocombustíveis. Estiveram presentes no Ato, além da Coordenadora do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do colegiado do Curso de Doutorado em Processos Biotecnológicos, ficou constituída pelos Professores Doutores Vanete Thomaz Soccol (UP), Saul Nitsche Rocha (UP), José Angel Rodriguez León (UP), Michele Rigon Spier (UFPR) e Carlos Ricardo Soccol (UFPR - orientador da tese).

Às 9h00, a banca iniciou os trabalhos, convidando o candidato **Luiz Gustavo Lacerda** a fazer a apresentação da Tese intitulada: "*Development of Bioprocess for the Production of Microbial and Algae Lipids from Starch Tubers*". Encerrada a apresentação, iniciou-se a fase de argüição pelos membros participantes.

Tendo em vista a tese e a argüição, a banca composta pelos professores Drª Vanete Thomaz Soccol, Dr José Angel Rodriguez León, Dr Saul Nitsche Rocha, Drª Michele Rigon Spier e Dr Carlos Ricardo Soccol declarou ao candidato APROVADO (de acordo com a determinação dos Artigos 59 a 68 da Resolução 65/09 de 30.10.09).

Curitiba, 28 de Dezembro de 2010



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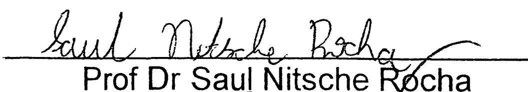
Profª Drª Michele Rigon Spier



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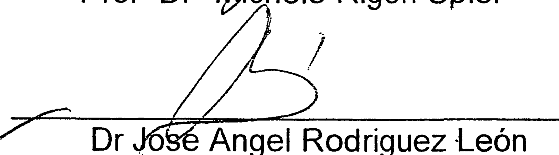

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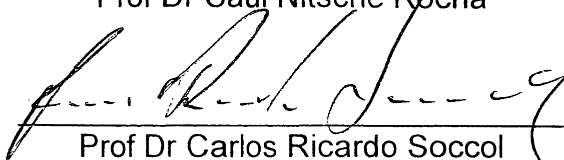

Prof Dr Carlos Ricardo Soccol

Universidade Federal do Paraná
Setor de Tecnologia
Curso de Doutorado em Processos
Biotecnológicos

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March, 23th 2000 – October,13th 2010†*

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ABSTRACT

Facing the problem of increasing energy demand, feasible fossil fuels substitutes have been studied. Some microbial strains are capable to accumulate large quantities of lipids also known as oleaginous microorganisms can use converted (into glucose) starch from cassava to lipid production that can be transesterificated to good quality biodiesel. The knowledge of starch content in amylaceous matter is very important once this residue can be used in further several industrial applications being a high value-added material**.** Cassava (*Manihot esculenta*) starchy fractions from integral flour and bagasse were submitted to starch content evaluation, acid hydrolysis optimization, thermal analyses and optical microscopy. The results reveals high good quality starch content in both studied materials. After that, in order we carried out a screening to select a potential biomass/lipid producer from seven different yeast strains using glucose from cassava hydrolysate. After a first selection, where strain A (LPB0035) presented better results a Plackett Burman planning was applied to identify factors presence that influence the lipid production yielding positively and KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4$, $CaCl_2$, urea and yeast extract were determined. Finally, a 2^3-factorial central composite design (2^3-CCRD) was used to evaluate optimum parameter levels for the production based on significative factors observed in previous screening and following concentrations were established (g/L): KH_2PO_4 (0,5), $MgSO_4$(0,30), $CaCl_2$(0,30) and urea (1,77). After testing process parameters, we found that *R. toruloides* LPB 0035 was capable to accumulate up to 56,7% (w/w) oil from hydrolysate of cassava tuber and its cell dry weight reached 44,3 g/L at the end of fed batch cultivation. Most of fatty acids obtained from *R. toruloides* LPB 0035 and *C. vulgaris* LPB 0033 were C16:0, C18:1 and C18:2. Finally, effluent from batch fermentation presented high biochemical demand oxygen (BOD) and chemical demand of oxygen (COD) values and was submitted to microalgae cultivation in order to reduce pollutants concentrations. Thus, after 17 days of cultivation using *C. vulgaris* LPB 0033 the final broth achieved more than 50% for both BOD and COD reductions. Biodiesel produced using studied process present suitable characteristics for official international biodiesel specifications.

INTRODUCTION

The energy consumption has increased quickly along the last century, following the population growth and due to the fact that more countries became industrialized. Fossil oil has been the biggest source for the supply of this increasing demand. There are been studied diverse techniques to estimate the current knowledge about the oil reserves and the reserves still not explored. Some researches research demonstrate the oil production probably will enter in decline before 2010, and also predicted that the oil production would fall from 25 billion barrels (current production) to approximately 5 billion barrels in 2050. Thus, considering the dependence of many countries on fossil fuels, the future consequences can be severe. To deal with these serious problems, such as deteriorated situation of the whole world energy supply, energy environment and energy security, alternative renewable biofuels are receiving great attention. Bioenergy is energy of biological and renewable origin, normally derived from purpose-grown energy crops or by-products of agriculture, forestry or fisheries. Examples of bio-energy resources are fuel wood, bagasse, organic waste, biogas and bio-ethanol. Moreover, it is the only renewable energy source that is available in gaseous, liquid and solid forms. One of the most prominent renewable energy resources is biodiesel, which is produced from renewable biomasss by transesterification of triacylglycerols, yielding monoalkyl esters of long-chain fatty acids with short-chain alcohol. It contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel. Biodiesel is been obtained worldwide from several sources including vegetables, animal and wasting oils. Some microorganisms, such as eukaryotic yeasts, molds, and algae, are known to produce triacylglycerols in their biomass (single-cell oils, SCOs) similar to plant oils. Some yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp. And *Lipomyces* sp. can accumulate intracellular lipids as high as 70% of their biomass dry weight using glucose or other simple sugars for the cultivation process. However, nowadays the costs of microbial oil production are currently higher than those of vegetable oil but there are many methods to drastically improve the feasibility of microbial oil production processes. In particular, the exploration of alternative sources of

carbohydrates as feedstock may greatly lower the costs. This research aim the development and establishment of a bioprocess that use cassava amylaceous content as glucose source for cultivation of *R. thoruloides* LPB 0035 strain in order to produce bio-oil. In addition, effluent from optimized fed batch process will be used to both produce olaeagionous biomass from microalgae *C. vulgaris* and to reduce pollutants present regarding BOD, COD and salts. Furthermore, the produced oil will be transesterificated into biodiesel and the quality of final product will be analysed.

OBJECTIVES

Genneral purpose:

This research work aimed to establish a bioprocess to produce lipids using as carbon source cassava tuber and its residue also known as bagasse.

Specific Purposes:

- Characterize the raw material to be used as carbon source in the bioprocess to obtain biodiesel.

-Establish an optimal condition for starch hydrolysis content in cassava.

- Select a potential strain capable to accumulate lipid and generate biomass from hydrolysated cassava starch.

- Study factors that may contribute to bioprocess yielding.

- Optimize suitable quantities of important chemical elements, nitrogen source, and C/N relation in lipid accumulation.

- Scalling up the bioprocess and verify good dO_2 value and nitrogen source.

- Use wastewater generated from fed-batch microbial fermentation for microalgae cultivation in order to reduce pollutants concentrations and generate lipids.

- Extract lipids from obtained biomass and transesterificate into biodiesel.

- Analyse qualitatively of lipids and biodiesels obtained.

CHAPTER I

LIPID RICH BIOMASS PRODUCTION USING HYDROLYZED STARCH AS CARBON SOURCE IN ORDER TO PRODUCE BIODIESEL – REVIEW.

ABSTRACT

Facing the problem of energy demand increasing, feasible fossil fuels substitutes have been studied. Some microbial strains are capable to bio-accumulate large quantities of lipids also known as oleaginous microorganisms can use converted (into glucose) starch from cassava to lipid production that can be transesterificated to good quality biodiesel. This research aim to show some aspects of cassava (*Manihot esculenta*) starch technology to providing integral root, bagasse and flour as carbon source to lipid rich biomass production.

Keywords: Cassava, hydrolysis, microbial biomass, lipids, biodiesel.

1.INTRODUCTION

Nowadays, the energetic alternative for the new oil crises lays on the agriculture, due to the production of raw material suitable for production of biofuels such as ethanol or biodiesel. Biofuels can be produced from raw material and residues whose main component is cellulose, but technologic restrictions still turn this process economically unfeasible.

Many cultures have the potential for biofuels production, especially those which store sugar and starch. From the storing sugar cultures, sugarcane completely dominates the market, especially in Brazil. In 2006 the culture occupied an area bigger than 7 million hectare (MAPA, 2008), from where more than 426 million tons of sugarcane were produced (UNICA, 2008). From this, approximately 17,9 billion liters ethanol were produced, considering both anhydrous and hydrated, and 30,7 million tons of sugar (MAPA, 2008). No doubt it is an impressive productive complex, but with an energetic matrix based on just one culture.

The main starch culture utilized for ethanol production, mainly in U.S., is corn, although its use presents some restrictions, such as the low energetic balance value (SHAPOURI *et al.*, 1995; SHAPOURI *et al.*, 2002), its vulnerability to environmental stresses and nessessity of high quantities of chemicals and enzymes and discussions regarding its use as food.

A security strategy for the energetic matrix is the use of others sources of raw material for biofuels production. In this context, sweet potato and cassava (starch cultures) can be used as alternatives sources for sugarcane. These are cultures with great productive and energetic potential, adapted to tropical and subtropical climates.

They are both characterized by their rusticity, use high energy productivity (carbohydrates) per area of cultivation, being one of the main food sources in countries with low levels of HDI (Human Development Index). Although they are also used as food, sweet potato and cassava are cultures which can be incorporated to great agricultural projects, requiring an adjustment on the handling of the culture with specific genotypes for energy, do not competing with food production. Furthermore, there is also the possibility of a mixed cultivation (with both cassava and sweet potato) without lowering any

levels of productivity, for each culture (MATTOS and SOUZA, 1987). From that, the starch productivity per hectare can be finally increased. The present research aims the development of a bioprocess of lipid rich biomass production using hydrolyzed starch as carbon source in order to produce biodiesel.

1.1 Cassava bagasse and integral cassava tuber

1.2 Cassava´s Industry

There has been an increased exploitation of organic residues from various sectors of agriculture and industries over the past few decades. Crop residues such as bran, husk, bagasse and fruit seeds are utilized as a potential raw material in bioprocesses as they provide an excellent substrate for the growth of microorganisms, supplying the essential nutrients to them (PANDEY; SOCCOL, 1998; PANDEY *et al.*, 1999; PANDEY *et al.*, 2000a). Their application in bioprocesses also offers advantages in bioremediation and biological detoxication of hazardous compounds (RAMACHANDRAN *et al.*, 2007).

Industrial processing of cassava is performed aiming at flour isolation (which generates more solid residues) and starch (which generates more liquid residues) from the tubers. Figure 1 shows the processing and mass balance of cassava tubers for starch isolation. Two types of wastes are generated: solid and liquid. Solid wastes include peels and bagasse. Liquid waste is also known as manipueira and its disposal is very serious to the environment (PANDEY *et al.*, 2000b). In Brazil, there are approximately 80 cassava processing establishments forming important specific production regions (VIEIRA *et al.*, 2002; CEREDA and VILPOUX, 2001).

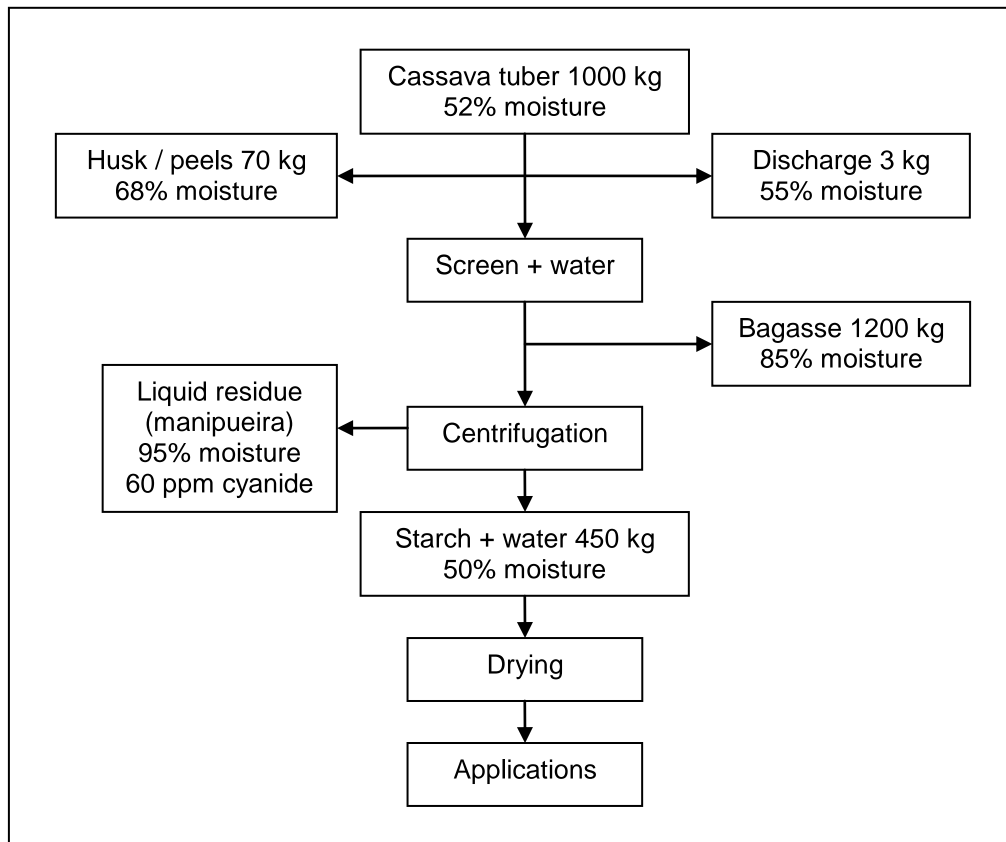

Figure 1 - Industrial processing of cassava (Source: Adapted from LIMA *et al.*, 2001; PANDEY *et al.* 2000b).

Cassava has the remarkable capacity of adaption to various agro-ecological conditions. It is also considered as a low-risk crop (PANDEY *et al.*, 2000a). Cassava is transformed into flour and starch during industrial processing, generating US$ 600 million in flour and 150 million in starch (FUKUDA, 2001). Normally, starch derived from cassava is destined the nourishing use, in meat products, pastas, desserts, breads, biscuits, soups and candies (CEREDA and VILPOUX, 2003).

Brazil used to be the greatest worldwide producer of cassava tubers. In the last years, it occupied second position as producer with 20,9 million tons, behind Nigeria that produces about 32,7 million tons. In Brazilian North and Northeast there is a predominance of familiar industries characterized by the artisan's work or small cassava facilities, whose product can be directed to consumption. Figure 2 shows the evolution of Brazilian cassava production. In Brazil, the domestic units are characterized by the use of familiar resources, thus they do not use modern technologies and represent a small part of the

overall production. However, big facilities process more than 400 ton tubers/day (GARNEIRO *et al.*, 2003).

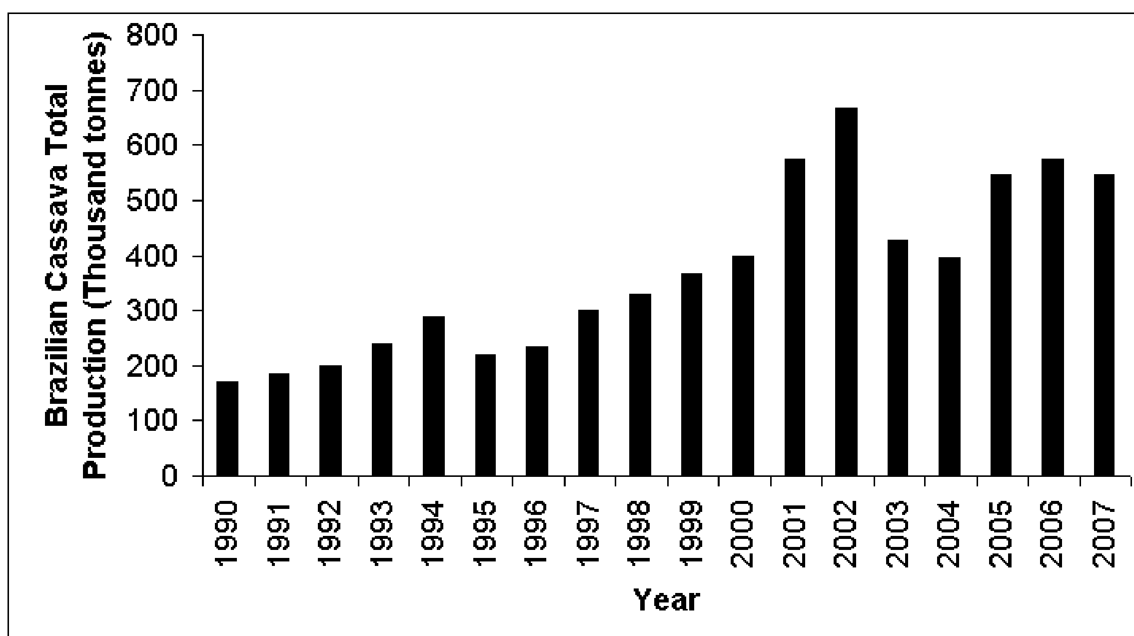

Figure 2 - Brazilian cassava production in thousand tons (Source: ABAM, 2009).

Starch sector is one of the most protected in the world, thus the majority of researches are developed inside the companies. This is prejudicial for the development of new companies and the small industries are destined to produce basic products, with simple technologies and low aggregated value products (CEREDA and VILPOUX, 2003).

The starch market has grown in the last years, leading to the search of products with specific characteristics that are required by the industry. The modified starch production is an alternative that is being developed in the last decades, with the objective to surpass one or more limitations of native starches and thus to increase the utility of this polymer in industrial applications (LEONEL; JACKEY; CEREDA, 1998; WURZBURG, 1986).

There is serious concerning about the losses of starch, and the potential uses of this residue are not exploited by the Brazilian cassava facilities. There are potential applications for the bagasse and the technological use of the residues must be determinative for a differentiation in a next future (PANDEY *et al.*, 2000b). Actually, many possibilities had been and are being explored for the destination of this residue, focusing on potential uses dictated by the market

situation. Regarding researches in small scale, the main objectives include the use of bagasse as supplement for bovine feeding (OSPINA, 1998), production of coal, packing (VICENTINI, CASTRO and CEREDA, 1999), the alimentary use as staple fiber source (25) and finally the use as substrate for alcoholic fermentation aiming at the production of bio-ethanol for pharmaceutical, beverage and bio-fuel uses (LEONEL, CEREDA and ROAU, 1999; LEONEL; CEREDA, 1998).

Cassava processing facilities, as well as all the industries, are affected by the economy changes, what compels them to manage productive resources in a dynamic way. Beyond the concerning on efficiency of starch extraction, the challenge is to add value and viable destination to the residues, turning them into co-products.

1.3 Cassava tuber

Cassava, represented in Figure 3, is considered to have originated in Venezuela during 2700 b.C. (SOCCOL, 1996). It was introduced in Africa during the 16th century and from there into Asia during the 18th century. It is a bushy plant producing tubers, made up of an aerial part and an underground part. The aerial part can be as high as 2-4 m with a trunk and branches on it. The underground part consists of two types of roots: the ones responsible for the plant nutrition, and the others with axial disposition surrounding the trunk. These are called tubers and are the edible parts of the plant. Each plant may have 5-20 tubers, and each tuber may attain a length of 20-80 cm and a diameter of 5-10 cm. The fresh weight of each tuber normally may vary from a few hundred grams up to 5 kg (PANDEY *et al.*, 1998).

Figure 3 - Cassava tuber and its aerial parts (Source: FAO, 2007).

Cassava has the remarkable capacity to adapt to various agro-ecological conditions. It is also considered as a low-risk crop. In view of its drought-resistant nature and non-requirement of any specific growth conditions, much attention has been paid in the last years to its agricultural aspects, for increasing its production all over the world, which has been well achieved (CARTA *et al.*, 1999).

1.4 Cassava bagasse

The processing of cassava tubers for the large-scale production of starch results in solid and liquid wastes. The fibrous slurry constitutes about 15-20% of the cassava chips/tuber processed, which contains around 50-70% starch on dry weight basis. Cassava bagasse or thippi, which is generally discarded to the environment without any treatment, causes serious concern about environmental pollution in areas where the starch industries are located (JYOTHI *et al.*, 2005).

The amount and the quality of these residues are functions of factors such as plant variety (species), cultivation process, machinery used, period after the harvest and many others. An important residue of the processing of cassava is the bagasse, the waste material of the root containing part of the starch that was not previously extracted. The raised amount of bagasse

generated in the facilities makes this material a problem during the harvest due to the difficulty in its transport and storage. This situation makes the use of technologies to prevent environmental impact to the industrial areas necessary (LACERDA *et al.*, 2009). Table 1 shows the composition of cassava bagasse as determined by various authors and different cassava processing units.

Table 1- Cassava bagasse constituents (dry basis) found in the literature.

Constituent	SOCCOL (1994)	CEREDA (1994)	STERTZ (1997)	VANDENBERGHE (1998)	CARTA (1999)	JYOTHY *et al.* (2005)
Moisture	5.02	9.52	10.70	11.20	9.78	5.87
Protein	1.57	0.32	1.60	1.61	1.51	NA
Lipids	1.06	0.83	0.53	0.54	0.54	NA
Fibers	50.55	14.88	22.20	21.10	16.87	29.13
Ash	1.10	0.66	1.50	1.44	1.62	NA
Sugars (Starch)	40.50	63.85	63.40	63.00	66.00	45-55.00

NA - Not available.

Because of its low ash content, cassava bagasse could offer numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ashes, respectively, for usage in bioconversion processes using microbial cultures. In comparison to other agricultural residues, cassava bagasse can be considered as a rich solar energy reservoir due to its (cassava's) easy regeneration capacity. When compared with sugar cane bagasse, it offers advantages, as it does not require any pretreatment and can be easily attacked by microorganisms (PANDEY *et al.*, 2000b).

1.5 Cassava processing and starch hydrolysis

Starch consists primarily of branched and linear chains of glucose molecules, named as amylopectin and amylose, respectively. Amylose is essentially a linear molecule with a few branches, whereas amylopectin (Figure 4) is a highly branched molecule (ALVES *et al.*, 2007).

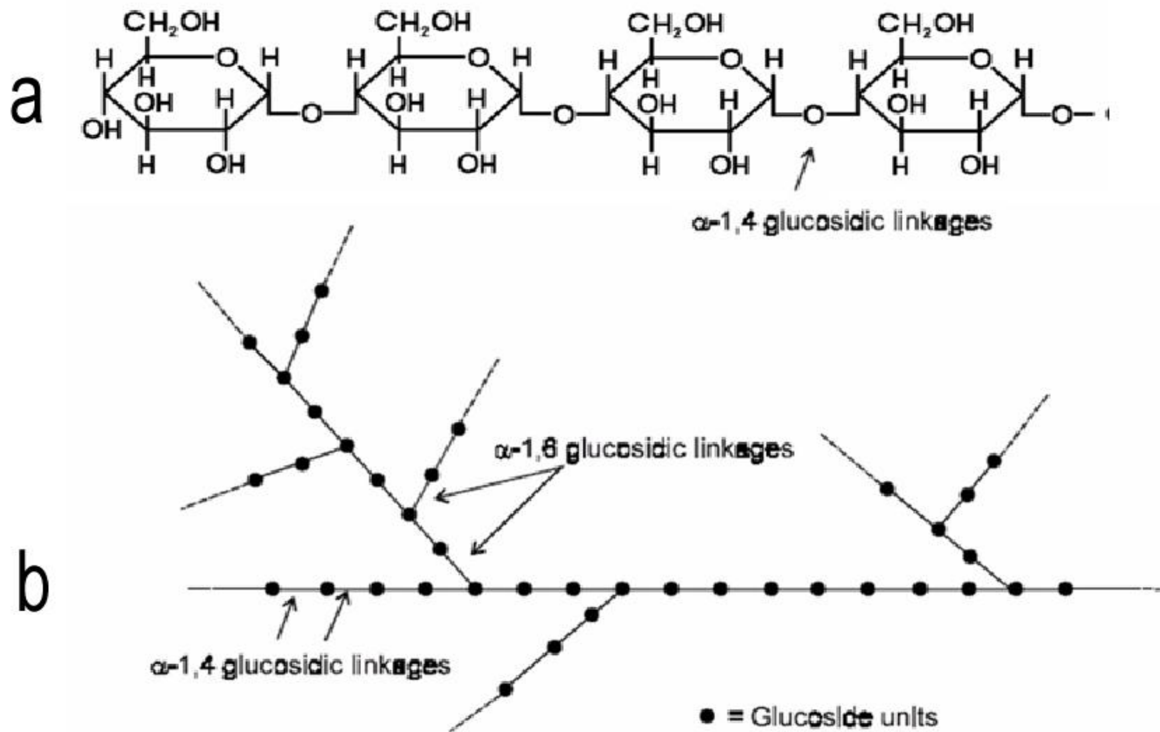

Figure 4 - (a) Amylose (linear) and (b) amylopectin representation with α-1,4 and α-1,6 linkages (Sources: LACERDA, 2006; JACQUES; LYONS; KELSALL, 1999).

To produce ethanol from starch it is necessary to break down the chains of this carbohydrate in order to obtain glucose syrup, which can be converted into ethanol by yeasts. This type of feedstock is the most utilized for ethanol production in North America and Europe. Corn and wheat are mainly employed with these purposes. In tropical countries, other starchy crops as tubers (e.g. cassava) can be used for commercial production of fuel ethanol (CARDONA; SANCHEZ, 2007).

1.6 Reducing sugars recovering from starchy matter

Studying the recovering of reducing sugars from cassava bagasse, WOICIECHOWSKI *et al.* (2000) observed good yields from both acid and enzymatic hydrolyses. The yield of the acid hydrolysis achieved 62.4g of reducing sugars from 100g of cassava bagasse containing 66% starch. It represented 94.5% of reducing sugars recovery. The yield of the enzymatic

hydrolysis was 77.1g of reducing sugars from 120g of cassava bagasse, which represented 97.3% of reducing sugars recovery.

Despite the fact that enzymatic hydrolysis has environmental claims, in the same study a batch of acid hydrolysis required 10 min, plus the time to heat and cool the reactor, and a batch of the enzymatic hydrolysis needed 25 h and 20min, plus the time to heat and to cool the reactor. Finally, the acid hydrolysis of 150kg of cassava bagasse demanded US$ 34.27, and the enzymatic hydrolysis of the same amount of cassava bagasse required US$ 2470,99.

CARTA *et al.* (1999), studying the hydrolysis of cassava bagasse using enzymes, also observed good yields using α-amylase, amyloglucosidase and cellulase. However, this process can be expensive mainly due to the price of the enzymes.

Considering the role of cassava in coutries facing serious poverty, the increase in production could be a very important achievement, justifying the enlargement in research financing. However, due to the substantial volume of fresh cassava, increase production does not necessarily mean trade. An economic barrier is the uncertainty of cassava supply and the strong competition of alternative products.

1.7 Starch technology

Starch present in roots and cereals must be converted into sugar before fermentation by yeasts. Starch hydrolysis or saccharification can be conduced by acid or enzymatic pathway, in continued or discontinued processes. Acid hydrolysis shortens time of starch saccharification, but there are a lot of restrictions for its use, such as partial sugar wrack and risk of non-fermentable sugar formation. Enzymatic hydrolysis, on the other side, demands trained technicians, high cost equipment and utilizes enzymes from vegetable or microbial sources, such as α-amylase e amyloglucosidase (VENTURINI FILHO and MENDES, 2003).

The idea of using raw material based in starch, especially sweet potato, for biofuel production is not new. A study published in 1909 where the productivity and percentage of dry root material on the ethanol yield were evaluated, being suggested that the root productivity would be the main factor

(KEITT, 1909). However, when the genotypes have different starch content, it can be seen that the starch has bigger contribution to ethanol yield (BOSWELL, 1944). War II (NEELY, 1997).

Besides the high potential for biofuel production, especially cultures with high root starch content, subproducts derived from fermentation process have suitable characteristics for animal feed increasing economics results of processing units (VENTURINI FILHO and MENDES, 2003).

1.8 Lipid rich biomass

According to Pandey (2000a) the use of vegetable oils as alternative renewable fuel competing with petroleum was proposed at the beginning of the 1980's. Nowadays, we have several vegetables sources of renewable crops rich in oils as described in Table 2. Vegetable and animal oils/fats represent potentially inexhaustible source of bio-energy, with energy content close to that of diesel fuel. However, there is no possibility to satisfact the World´s energetic matrix using only biodiesel from vegetable or animal sources. Other alternative sources of fat/oils such as algae and microrganisms have been studied in the last years in order to represent potential use as biodiesel feedstock.

Table 2 – World´s vegetable oil plantation areas and oil production in 2005 (Source: PANDEY, 2009).

Origin	Oil Production (Million Tons)	Leading Countries	Plantation Area (million ha)
Soybean	29.1	United States and Brazil	78.6
Palm	29.6	Malaysia and Indonesia	8.9
Rapessed	14.7	Europe	27.8
Sunflower	9.2	France and Italy	19.5
Coconut	4.5	Philippines	10.4

1.9 Microorganisms capable to accumulate lipids

Microorganisms that accumulate large quantities of lipids (oleaginous microrganisms) have been the object of research for many years and they are also as known as single cell oil (SCO). Their physiology has been studied in detail, in classical reports and their potential industrial applications have been

described in technical papers (AGGELIS; SOURDIS, 1997). The bulk of the natural oil made by oilseed crops is in the form of triacylglycerols (TAGs). TAGs consist of three long chains of fatty acids attached to a glycerol backbone. Some microrganisms species for instance can produce more than 50% of their body weight in the form of TAGs (Table 3). Thus, selected microrganisms represent an alternative source of biodiesel, one that does not compete with the existing oilseed market (RATLEDGE; WYNN, 2002).

Table 3 - Oil content of some microorganisms (Source: Meng *et al.*, 2009)

Microorganism	Oil content (% dry basis)	Microrganism	Oil content (% dry basis)
Microalgae		**Yeast**	
Botryococcus braunii	25-75	*Candida curvata*	58
Cylindrotheca sp.	16-37	*Cryptococcus albidus*	65
Nitzschia sp.	45-47	*Lipomyces starkeyi*	64
Schizochytrium sp.	50–77	*Rhodotorula sp.*	72
Bacterium		**Fungi**	
Arthrobacter sp.	>40	*Aspergillus oryzae*	57
Acinetobacter calcoaceticus	27-38	*Mortierella isabellina*	86
Rhodococcus opacus	24-25	*Humicola lanuginosa*	75
Bacillus alcalophilus	18-24	*Mortierella vinacea*	66

The costs of microrganisms oil production are currently higher than those from vegetable oil. However, there are many methods to drastically improve the techno-economics of microbial oil production processes. In particular, the exploration of residues as feedstock may greatly lower the costs. Process engineering that leads to a higher lipid production rate and cellular lipid content may also contribute in this concern. Different fermentation types, including fed-batch, have been used to optimize oleaginous microrganisms yielding. Known factors controlling lipid accumulation in oleaginous microrganisms are the composition of the medium, such as the C/N ratio and nitrogen source and the culture conditions, such as temperature and dissolved oxygen (KAMISAKA; NODA; YAMAOKA, 2004; KIMURA, K.; YAMAOKA, M.; KAMISAKA, Y, 2006; LI; ZHAOB; BAI, 2007).

Some yeast strains, such as *Rhodosporidium sp., Rhodotorula sp.* , and *Lipomyces sp.* can accumulate intracellular lipids as high as 70% of their biomass dry weight. The majority of those lipids are triacylglycerol (TAG) contained long-chain fatty acids that are comparable to conventional vegetable oils (LI; ZHAOB; BAI, 2007).

1.10 Wastewater treatment

Industrial wastewaters commonly present high biological and chemical hazard compounds. Thus, before their disposal, it is necessary to treat effluents by physical- or chemical-treatment processes. These include chemical coagulation/flocculation, ozonation, oxidation, ion exchange, irradiation, precipitation and adsorption. Some of these techniques have been shown to be effective, although they have limitations. Among these are: excess amount of chemical usage, or accumulation of concentrated sludge with obvious disposal problems; expensive plant requirements or operational costs; lack of effective color reduction; and sensitivity to a variable wastewater input (CLARKE; ANLIKER, 1980; SUMATHI; MANJU, 2000; ROBINSON *et al.*, 2001; AKSU;TEZER, 2005). Potentially environmental hazardous compounds such as metals, organic residues, CO_2 are usually generated through fermentation processes. Their mitigation through microalgae has attracted much attention as a strategic alternative that associates both environmental and economical interests (SIDNEY *et al.*, 2010). An example of photobioreactor for microalgal culture and CO2 reduction is presented schematically in Figure 5.

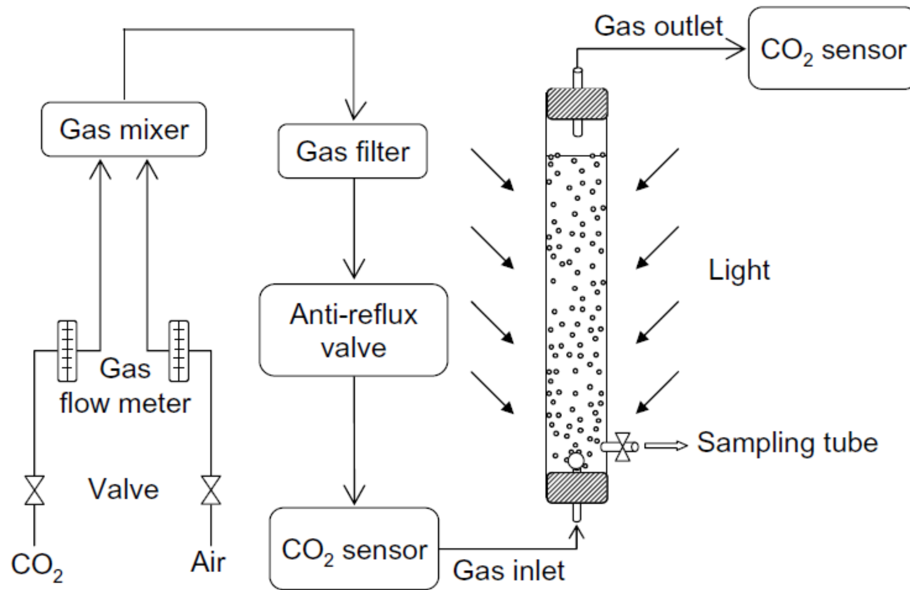

Figure 5 - Schematic diagram of the photobioreactor for the experiments on CO_2 reduction for batch microalgal cultures (Source: CHIU et al, 2008).

The utilization of industrial gaseous and/or liquid residues is becoming a reality in microalgae cultures in view that the major barrier in industrial cultivation of microalgae is the cost of the media for cultivation. Therefore, knowledge of residue composition, microalgae metabolic pathways and nutritional needs plays a central role in processes development (SIDNEY *et al.*, 2010).

The special surface properties of algae, bacteria and fungi enable them to adsorb different kinds of metalic and organic pollutants from solutions. ‘‘Biosorption’’ term is used to indicate a number of metabolism-independent processes (physical and chemical adsorption, ion exchange, complexation, chelation and microprecipitation) taking place essentially in the cell wall (AKSU; TEZER, 2005). *C. vulgaris* has been reported for Its ability to reduce organic and inorganic pollutants levels from wastewaters. Furthermore this microalgae is capable to accumulate good quality lipid in order to produce biodiesel (CLARKE; ANLIKER, 1980; SUMATHI; MANJU, 2000; ROBINSON *et al.*, 2001; AKSU; TEZER, 2005; AFKAR et al, 2010; SIDNEY *et al.*, 2010).

1.11 Biodiesel

Rudolph Diesel first used peanut oil (which is mostly in the form of TAGs) at the turn of the century to demonstrate his patented diesel engine (PETERSON, 1986). Nowadays, it is well known natural oils, it turns out, are too viscous to be used in modern diesel engines.

During the 1980s, a chemical modification of natural oils was firstly introduced to bring the viscosity of the oils within the range of current petroleum diesel (BRUWER *et al.*, 1980). By reacting these TAGs with alcohols (a chemical reaction known as "transesterification" (Figure 6) in the oleochemicals industry), it is possible to create a chemical compound known as an alkyl ester (MARKLEY, 1961), more generically known as biodiesel. Its properties are very close to those of petroleum diesel fuel.

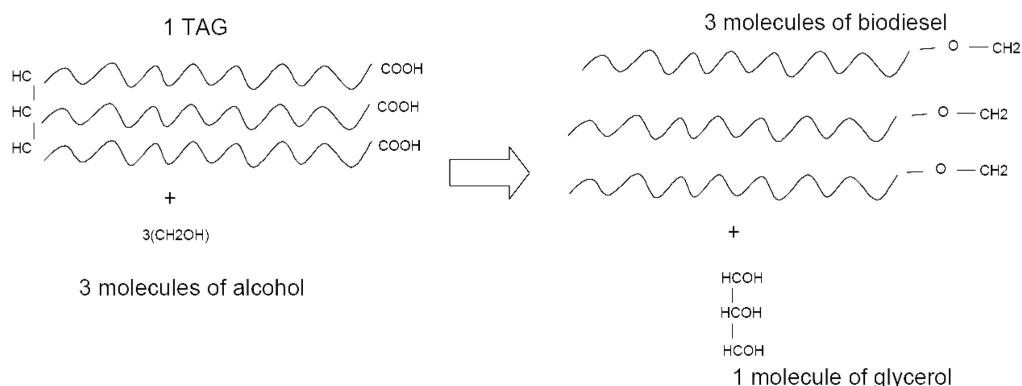

Figure 6 - TAG transesterefication into biodiesel and glycerol.

Chemically, biodiesel is a diesel-equivalent fatty acid alkyl esters produced through transesterification reaction of triacylglycerol lipids with methanol or ethanol. Among the many advantages of biodiesel fuel including the following: it is safe for use in all the conventional diesel engines; it offers the same performance and engine durability as petroleum diesel fuel; it is nonflammable and nontoxic and reduces tailpipe emissions, visible smoke, and noxious fumes and odors as described in Table 4. It is better than diesel fuel in terms of the sulfur content, flash point, aromatic content, and biodegradability (DERMIBAS, 2003;CHEN, X. *et al.*, 2009).

Between 1991 and 2001, world biodiesel production grew steadily to approximately 1 billion liters. The governments around the world have instituted various policies to encourage development of the industry, and new capacity in North America, South-east Asia and Brazil has begun to come on stream at a brisk rate. As a result, between 2001 and 2007, biodiesel production will have grown almost tenfold, to 9 billion liters (DEMIRBAS, 2009).

Table 4 - Average changes in mass emissions from diesel engines using biodiesel mixtures relative to the standard diesel fuel (%)(DEMIRBAS, 2009).

Mixture	**CO**	**NOx**	**SO_2**	**Particular matter**	**Volatile organic compounds**
B 20	-13.1	+ 2.4	- 20	- 8.9	- 17.9
B 100	- 42.7	+ 13.2	- 100	- 55.3	- 63.2

The feedstock accounts for 70% to 80% of total cost of biodiesel production, and clearly is the key factor to evaluate when considering the competitiveness of biodiesel with petroleum-based diesel fuel.

The production of worldwide biodiesel regards in various researches on renewable lipids sources including vegetable oils, animal fats and wasting oils (AGGELIS, *et al.*, 1995). Engine manufacturers and biodiesel plants in different parts of the world use slightly different standards for biodiesel. Virtually all modern diesel engines warranties permit the use of biodiesel provided it meets certain specifications as showed in Table 5.

Table 5 – Biodiesel specifications according to different legislations.

Trial	ANP Specification (2008)	European Specification EN 14214	USA Specification ASTM 6751
Specific gravity at 20°C	850-900	-	-
Kinematic viscosity at 40°C	3.0-6.0	3.5-5.0	1.9-6.0
Water	Max. 500 mg/kg	Máx. 500 mg/kg	Max. 0.05 % vol.
Contamination	Max. 24 mg/kg	Max. 24 mg/kg	-
Flash point	Min. 100 °C	Min. 101 °C	Min. 130 °C
Carbon residue 100%	Max. 0.05 %	Máx. 0.01 %	Max. 0.05 %
Total sulphur	Max. 50 mg/kg	Max. 10 mg/kg	Max. 0,05 %
Phosphorus	Max. 10 mg/kg	Max. 10 mg/kg	Max. 0,01 %
Ester content	Min. 96,5 % mass	Min. 96,5 % mass	-
Free glycerin	-	Max. 0.02% mass	Max. 0.02% mass
Total glycerin	-	Max. 0.25 % mass	Max. 0.24 % mass
Monoglycerides	-	Max. 0.8 % mass	-
Diglycerides	-	Max. 0.2 % mass	-
Triglycerides	-	Max. 0.2 % de massa	-
Methanol or ethanol	-	Max. 0.2 %	Max. 0.2 %
Oxidation stability at 110°C	Min. 6 hours	Min. 6 hours	Min. 3 hours
Sulfated ashes	Max. 0.20 % mass	Max. 0.20 % mass	Max. 0.20 % mass
Acid Index	Max. 0.5 mg KOH/g	Max. 0.5 mg KOH/g	Max. 0.5 mg KOH/g
Cold filter plugging point	Max. 19	-	
Iodine index	-	Max. 120	-

In South East Asia, Europe, United States and China, palm oil, rapeseed oil, transgenic soybeans and wasting oil were used to produce biodiesel, respectively. However all plant oil materials require energy and land for

sufficient production of oilseed crops. Likewise, animal fat oils need to feed these animals. Microorganisms have often been considered for the production of oils and fats as an alternative to agricultural and animal sources (MENG *et al.*, 2009).

According to MENG *et al.* (2009), a fossil fuel substitute must be not only an alternative fuel having superior environmental benefits over the fossil fuel it displaces but be economically competitive with it, and be producible in sufficient quantities to make a meaningful impact on energy demands and also provide a net energy gain over the energy sources used to produce it.

CONCLUSION

The opportunities for the future for biodiesel include improvements in the conversion technology, expanding the amount of available feedstock, and adding value to the glycerol by-product as raw and pharmaceutical grade. Besides, carbon, nitrogen sources and other factors of biomass production from cheap and alternative raw materials can be the key to biodiesel production from oleaginous microbial biomass feasibility.

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CHAPTER II

CHARACTERIZATION OF AMYLACEOUS MATTER FROM CASSAVA (*MANIHOT ESCULENTA*) TUBER: INTEGRAL FLOUR AND BAGASSE.

ABSTRACT

The knowledge of starch content in amylaceous matter is very important once this residue can be used in further several industrial applications being a high value-added material**.** Cassava (*Manihot esculenta*) starchy fractions from integral flour and bagasse were submitted to starch content evaluation, acid hydrolysis optimization, thermal analyses and optical microscopy. The results revealed high good quality starch content in both studied materials. Based on acid hydrolysis determination, cassava bagasse and integral flour presented estimated starch content of 84.44% and 89.57% respectively. The use of HCl 1N at 121°C during 12 minutes reveals good hydrolysis condition. The average granule size of cassava starch was about 10 - 20µm. Thermogravimetry curves showed two main losses under synthetic air and differential scanning calorimetry was used to determine starch gelatinization (62°C) and melting point of material (170°C).

Key-words: cassava, experimental planning, bagasse, thermal-analysis, hydrolysis.

1. INTRODUCTION

Starch market comes growing in the last years, leading to the search of products with specific characteristics that support the requirements of the industry. The modified starch production is an alternative that is in continous development to surpass one or more limitations of native starches and thus to increase the utility of this polymer for industrial applications (LACERDA *et al.*, 2008a; LEONEL; CEREDA; ROAU, 1999; RUDNIK *et al.*, 2006; WURZBURG, 1986). Furthermore, there has been an increased exploitation of organic residues from various sectors of agriculture and industries over the past few decades. The use in biotechnological processes of crop residues such as bran, husk, bagasse, and fruit seeds regards as potential raw material in bioprocesses as they provide excellent substrates for the growth of microorganism supplying the essential nutrients to them (PANDEY; RADHAKRISHNAN, 1992; PANDEY *et al.,* 1994; PANDEY; SOCCOL, 1998; PANDEY *et al.*, 2000). The knowledge of starch content in amylaceous matter is very important once this residue can be used in further several industrial applications being a high value-added material. This study was performed to evaluate two usual methods for starch content determination in material, evaluate optimal hydrolysis conditions using 2^3- composite central rotational design (2^3-CCRD) as well structural characterization using several analytical methods.

2. MATERIALS AND METHODS

2.1 Samples

Cassava bagasse was obtained from a starch industry (Comercial Agricola Anhumai) located in the Northwest Region of Parana State - Brazil. Cassava (*Manihot esculenta*) was acquired from a local market (Curitiba, Parana State - Brazil). In order to obtain cassava integral flour, the material was grinded and dried in an oven during 48h at 100˚C, finally screened in order to obtain particles < 1mm. The enzyme α-amylase, from *Bacillus licheniformis* (Termamyl

240L), was acquired from Novozymes (Bagsvaerd, Denmark). Other reagents (i.e. sulfuric acid) were of analytical grade.

2.2 Hydrolysis

In order to solubilize starch content of samples, it were adopted chemical and enzymatic hydrolyses as following description. For both situations, the experiments were conducted in triplicate.

2.2.1 Chemical Hydrolysis

Chemical hydrolysis was carried out following method, adapted from WOICIECHOVSKI *et al.* (2002), where 2.0g of dry cassava bagasse (moisture content of 10 %) was added to 20.0 mL of distilled water and 2.0 mL of sulfuric acid 98% in an Erlenmeyer flask covered with aluminum foil paper and rubber band. Hydrolysis reaction was carried out in a water bath at approximately 98°C during 20min. After material cooling to room temperature (about 25°C), the pH was adjusted to 7.0 using sodium hydroxide 0.5 mol/L. The total volume was completed to 100mL with distilled water in a calibrated volumetric flask (±0.1 mL). The aqueous medium was filtered in Whatman filter paper and then an aliquot of 2.0mL was separated. Reducing sugar content of the filtered solution, after a 1:10 (solution:water) dilution, was estimated with a spectrophotometer SHIMADZU UV-1601 at 540nm following DNS method (MILLER, 1959; TASUN, 1970). This method involves spectrophotometric measurement of reducing sugar liberated from a known soluble starch medium by the action of amylase using dinitrosalicylic acid reagent at pH 6.9 and room temperature (MILAN *et al.*, 2008).

2.2.2 Enzymatic hydrolysis

In order to hydrolyse matter using α-amylase in excess, separated samples of 1.0g were added to 25mL of distilled water and 0.25mL of enzyme Termamyl 240L preparation in individual 150.0mL Erlenmeyer flasks. The pH was adjusted to 5.6 following enzyme data sheet instructions. After covering Erlenmeyer flask

using aluminum foil paper and rubber band, the sample was kept in a water bath at 90-95ºC during 25min. under continuous stirring. Sample was removed from the water bath and the pH of aqueous medium was adjusted to 9.0 using sodium hydroxide 0.5 mol/L in order to stop the enzyme action. The aqueous medium was filtered in Whatman 42 filter paper using a vacuum pump. Solid samples remaining on paper filter was dried in an oven at 60ºC during 48h, and then kept in a desiccator over anhydrous calcium chloride for further analyses.

2.3 Hydrolysis Optimization

Hydrolyses yielding under high pressure and temperature were investigated in order to achieve an optimum condition of starch conversion. 2^3-factorial central composite designs (2^3-CCRD) were carried out in order to identify optimum parameter levels of hydrolyses. The CCRD contained a total of 17 experimental trials that included eight trials for factorial points, six trials for axial points and three trials for replication of the central points. Central points provide additional degrees of freedom for error estimating, which increases power when testing the significance of effects. The distance of the axial points was ± 1.68, calculated from Equation 1:

$$\alpha=(2^n)^{1/4} \quad (1)$$

Where α is the distance of the axial points and n is the number of independent variables. All data were treated with STATISTICA® 5.0 from Statsoft Inc. All experiments were conducted using cassava integral flour at 121°C using an autoclave (Phoenix model Av 75). The effects of following variables: time, acid:starch mass relation and concentration of HCl as illustrated in Table 1. Experiments were conducted using grams as mass unit (i. e. at central point, it was used 4,0g of acid and 1,0g of flour) in glass flasks sealed with aluminum foils using rubber bands.

Table 1 - Variables and respective levels of hydrolysis optimization planning.

LEVELS	**-1.68**	**-1**	**0**	**1**	**1.68**
Time (min.)	4	5	12	17	20
Mass relation (acid: starch)	2.32:1	4:1	5:1	6:1	7.68:1
HCl (N)	0.32	0.66	1	1.34	1.68

We evaluate hydrolysis optimization using a complete experimental planning 2^3 with three repetitions at central point as showed in Table 2.

Table 2 - Matrix of complete experimental design 2^3 for hydrolysis studies.

Experiment	**Variables**		
	Time	Mass relation	HCl
1	(-) (5)	(-) (4:1)	(-) (0.66)
2	(+) (17)	(-) (4:1)	(-) (0.66)
3	(-) (5)	(+) (6:1)	(-) (0.66)
4	(+) (17)	(+) (6:1)	(-) (0.66)
5	(-) (5)	(-) (4:1)	(+) (1.34)
6	(+) (17)	(-) (4:1)	(+) (1.34)
7	(-) (5)	(+) (6:1)	(+) (1.34)
8	(+) (17)	(+) (6:1)	(+) (1.34)
9	(-1.68) (4)	0 (5:1)	0 (1.00)
10	(+1.68) (20)	0 (5:1)	0 (1.00)
11	0 (12)	(-1.68) (2.32:1)	0 (1.00)
12	0 (12)	(+1.68) (7.68:1)	0 (1.00)
13	0 (12)	0 (5:1)	(-1.68) (0.32)
14	0 (12)	0 (5:1)	(+1.68) (1.68)
15	0 (12)	0 (5:1)	0 (1.00)
16	0 (12)	0 (5:1)	0 (1.00)
17	0 (12)	0 (5:1)	0 (1.00)

The determination of yielding was made based on potential starch conversion based on total matter content. The model generated was analyzed using an analysis of variance (ANOVA).

2.4 Thermal Analysis

Thermal analysis TG (Thermogravimetry), DTA (Differential Thermal Analysis) and DSC (Differential Scanning Calorimetry) curves were recorded using a SHIMADZU TG 60 and DSC 60, with synthetic air flowing at 100 mL/min, and a heating rate of 10°C/min. and with mass samples of about 6 mg.

Alumina open sample holder and aluminum sealed crucibles were used for TG/DTA and DSC respectively. In order to study the gelatinization event, DSC studies were carried out. A 4:1 (water:starch) mixture was prepared and left overnight in order to equilibrate moisture content. TG is used to measure the mass loss either as a function of time (isothermal) or dynamic temperature and controlled atmosphere (MOTHÉ *et al.*, 2006). Endothermic and exothermic changes in a DSC curve indicate events or reactions such as glass transition, gelatinization and melting, occurring during DSC analysis (HABITANTE *et al.*, 2008).

2.5 Microscopy

Optical microscopy has been widely used by scientists and students as a useful tool to examine objects on a fine scale in order to get information relative to the morphology of the materials examined. After drying, samples of cassava bagasse untreated and treated by acid and enzyme were mounted on standard glass microscope slides. Microscopy analysis was carried out using an Olympus stereo microscope SZX9, with polarization filter and Cybernetic's Cool Snap Pro Color camera. The photographs were identified and scaled using Image Pro Plus.

3. RESULTS AND DISCUSSION

3.1 Acid hydrolysis

Acid hydrolysis has been used to modify starch for over 150 years (DUEDAHL-OLESEN; PEDERSEN; LARSEN, 2000) This process involves suspending starch in an aqueous solution of hydrochloric acid or sulfuric acid at certain temperatures. In the presence of a strong acid and heat, the glycosidic bonds between monosaccharides in a polysaccharide are cleaved. After hydrolysis using concentrated sulfuric acid as agent, from DNS method, it was obtained at 540 nm an optical density (OD) of 1.59 (±0.13). Following calibration curve made for DNS reagent (Figure 1), glucose concentration of sample was estimated following as Equation (2).

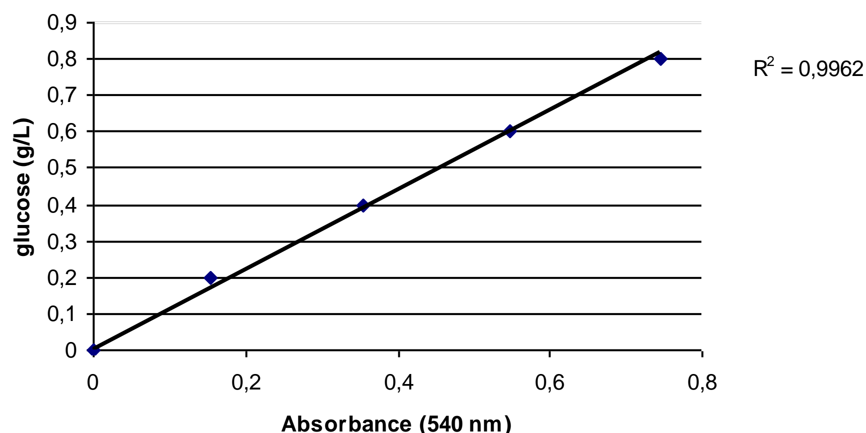

Figure 1 - Calibration curve of DNS reagent.

$$RS\left(\frac{g}{L}\right) = \left(.099 \times OD + 0.017\right) \times D \qquad (2)$$

Where RS is related to reducing sugars concentration in g/L, OD is optical density at 540 nm, D is dilution of original hydrolysis solution and other values are related to calibration slope from standard curve. Considering initial moisture content, It were studied 1.78g of cassava bagasse in dry basis (11.10% of moisture) and 1.79g of cassava flour (10.57% of moisture). Stoichiometrically, each 162g of starch, incorporates 18g of water during hydrolysis, producing 180g of glucose, thus there is a conversion factor equals to 1.11. In order to estimate starch content of studied sample it was used (Equation (3)).

$$SC_1(\%) = \frac{(100) \times IGC}{1.11} \qquad (3)$$

Where SC_1 is starch content, IGC is the initial glucose concentration and 1.11 is glucose-starch stoichiometric relation. Following the Equation (2), cassava bagasse and cassava flour estimated starch content were 84.44% and 89.57% respectively considering the studied samples. Acid hydrolysis is widely used as a method to determine starch (carbohydrate) content in a sample. However, degradation action is not specific to sugars and some fibrous matter is affected also. Based on reducing sugars, DNS method is not indicated when is needed to identify concentrations of specific carbohydrates with different molecular chain lengths (i.e. mono, di and trisaccharides).

3.2 Enzyme Hydrolysis

According to DUEDAHL-OLESEN, PEDERSEN AND LARSEN (2000) and SWINKELS (1985) α-amylases (Enzyme Classification 3.2.1.1) are endo-acting enzymes that randomly attack the internal α-D-(1.4) O-glycosidic linkages of starch except for those adjacent to the ends of the substrate and those in the vicinity of branch points. The end products are α-limit dextrins, which are branched saccharides not prone to further hydrolysis and malto-oligosaccharides of varying degrees of polymerization (DP), characteristic of the particular enzyme. Basically, starch content in this method was evaluated as difference among soluble fraction after hydrolysis and non soluble remaining material. Using enzyme bacterial α-amylase in excess, starch is converted into soluble fractions of maltose and other shorter chains of glucose units also known as dextrins. After drying the remaining material, final mass of 0.10g (±0.01) and 0.11 (±0.01) were observed concerning cassava bagasse and cassava flour respectively. Considering moisture contents of untreated and hydrolyzed sample showed in Table 1, starch content in dry basis was calculated by the (Equation3).

$$SC_2(\%) = (100) \times (FM - M) \qquad (3)$$

Where SC_2 is estimated as starch content in sample, FM is final mass or material mass remained on filter paper and M is related to moisture content (i.e 0.89 when 11 % of moisture). Regarding this method, cassava bagasse sample studied by enzyme hydrolysis has approximately 78.80% and cassava flour has 87.32% of starch. It is important to note that the use of enzymes can provide besides soluble fraction, the dietary fiber content. Actually, solid product from hydrolysis is mainly fiber and other compounds such as minerals and eventually lipids and others.

FAITHFULL (1990), studying the determination of starch content in potatoes (*Solanum tuberosum*) by enzyme hydrolysis observed several disadvantages comparing to the acid hydrolysis procedure. The extraction and hydrolysis process is slower, especially using amyloglucosidase, enzyme activity may

vary, reagents are more expensive, and complete hydrolysis is more difficult. Its main advantage is that it is specific, not affecting the cellulosic polysaccharides in the cell walls.

It is important to mention that both methods studied are very quick, useful and low cost procedures. However they have experimental limitations.

3.3 Hydrolysis optimization

Considering 89.57% of starch content and 11.57% of moisture content in cassava flour, it was possible to estimate conversion yielding by DNS method (MILLER, 1959) and following stoichiometry of starch hydrolysis as described previously during starch content (acid hydrolysis) studies. Thus, Table 3 shows results obtained for experiments for 2^3 CCRD. Estimated effects for each factor as weel their relations are presented in Table 4. Statistical parameters of t-test and p value were used to confirm significance of studied factors**.**

Table 3 - Starch conversion yielding of cassava starch present in integral flour.

Experiment	**Variables**			**Yield (%)**
	Time	Mass relation	HCl	
1	(-)	(-)	(-)	**90.12**
2	(+)	(-)	(-)	**89.23**
3	(-)	(+)	(-)	**89.75**
4	(+)	(+)	(-)	**90.56**
5	(-)	(-)	(+)	**90.00**
6	(+)	(-)	(+)	**89.23**
7	(-)	(+)	(+)	**91.45**
8	(+)	(+)	(+)	**91.25**
9	(-1.68)	0	0	**93.47**
10	(+1.68)	0	0	**92.12**
11	0	(-1.68)	0	**88.21**
12	0	(+1.68)	0	**94.98**
13	0	0	(-1.68)	**89.41**
14	0	0	(+1.68)	**92.12**
15	0	0	0	**94.78**
16	0	0	0	**95.56**
17	0	0	0	**95.01**

According to obtained results best conditions for hydrolysis yielding were determined by experiments 15, 16 and 17 (central point). On the basis of ANOVA, a second order model was obtained (Equation 4) describing hydrolysis

yielding as a function of hydrolysis time at 121°C, mass relation and HCl concentration. Based on t-test, present model is predictive. Calculated F value is higher than Tabled F.

$$y=95.20-1.1124x_1^2+1.1580x_2-1.5366x_2^2+0.2568x_3-1.830x_3^2 \quad (4)$$

Where y is provided hydrolysis yielding, x_1 is hydrolysis time (quadratic), x_2 is acid: starch relation (linear and quadratic) and x_3 regards to HCl concentration (linear and quadratic). Based on F-test, model is predictive once calculated f-value is higher than critical F-value. Predictive value versus observed value (Figure 2) resulted in a determination coeficient of 0.80 wich means that studied variables explain 80% of observed effects.

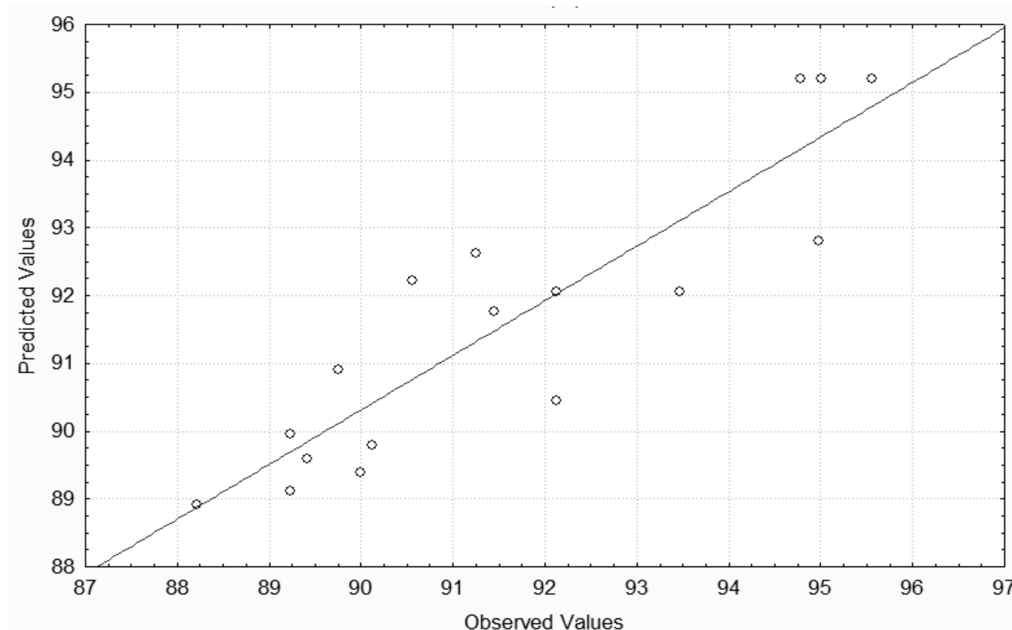

Figure 2 - Observed versus predicted values relation.

Pareto Diagram analysis (Figure 3) showed that, variables, HCl, Mass relation and time presented significant effects on starch hydrolysis. However, only mass relation (linear) presented a significative and positive effect. On the other hand, variable HCl (quadratic) mass relation (quadratic) displayed negative effects suggesting that the acid hydrolysis prevails at lower concentrations of

acid and mass relation. Time, according to Pareto´s diagram, is at the limit, thus, 12 min. is an ideal hydrolysis condition for this experiment.

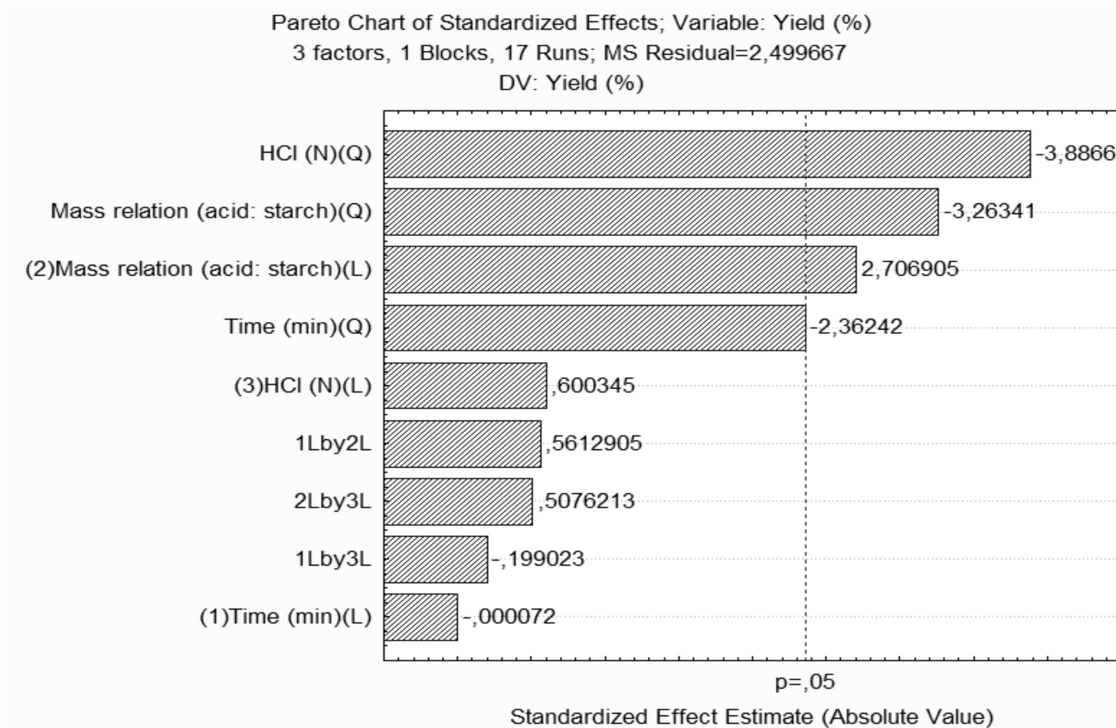

Figure 3 - Pareto Diagram for hydrolysis yielding optimization.

ANOVA analysis (Table 4) variance was used to estimate the adjusted model. R squared equals to 0.80516 and It shows variables can explain more than 80% of established model.

Table 4 - Variance analysis (ANOVA) of factors.

Variation source	SS	df	MS	F	p
(1)Time (min)(L)	0.00000	1	0.00000	0.00000	0.999945
Time (min)(Q)	13.95069	1	13.95069	5.58102	0.050163
(2)Mass (acid: starch)(L)	18.31590	1	18.31590	7.32734	0.030334
Mass (acid: starch)(Q)	26.62113	1	26.62113	10.64987	0.013799
(3)HCl (N)(L)	0.90092	1	0.90092	0.36041	0.567196
HCl (N)(Q)	37.75918	1	37.75918	15.10569	0.006002
1L by 2L	0.78751	1	0.78751	0.31505	0.592106
1L by 3L	0.09901	1	0.09901	0.03961	0.847902
2L by 3L	0.64411	1	0.64411	0.25768	0.627318
Error	17.49767	7	2.49967		
Total SS	89.80555	16			

Effect Estimates; Var.:Yield (%); R-sqr=,80516; Adj:,55465 (Spreadsheet1) 3 factors, 1 Blocks, 17 Runs; MS Residual=2,499667 DV: Yield (%)

Surface response (Figures 4, 5 and 6) show coded model described by Equation 4. It is noted that maximum points related to hydrolysis yielding are close to central point. Present model predicts maximum yield (95.11%) regards to mass relation of (5:1), HCl concentration of 1N and hydrolysis time of 12 min. at 121°C.

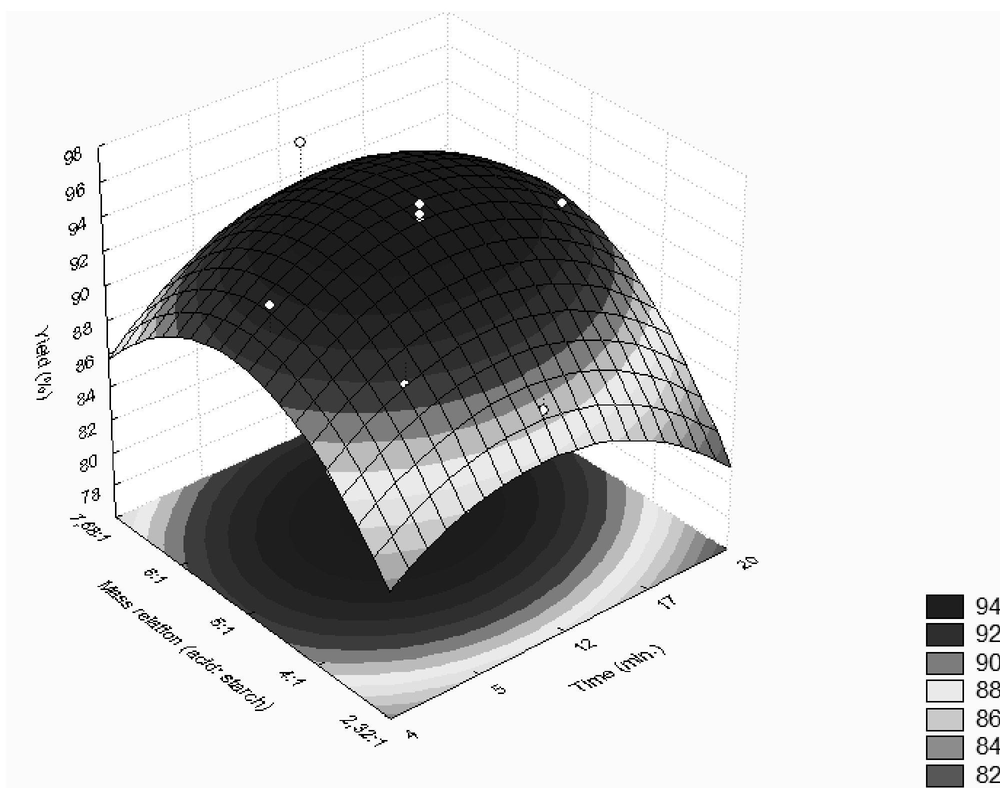

Figure 4- Response surface curve of hydrolysis yielding showing optimal range and values of Mass relation and Time.

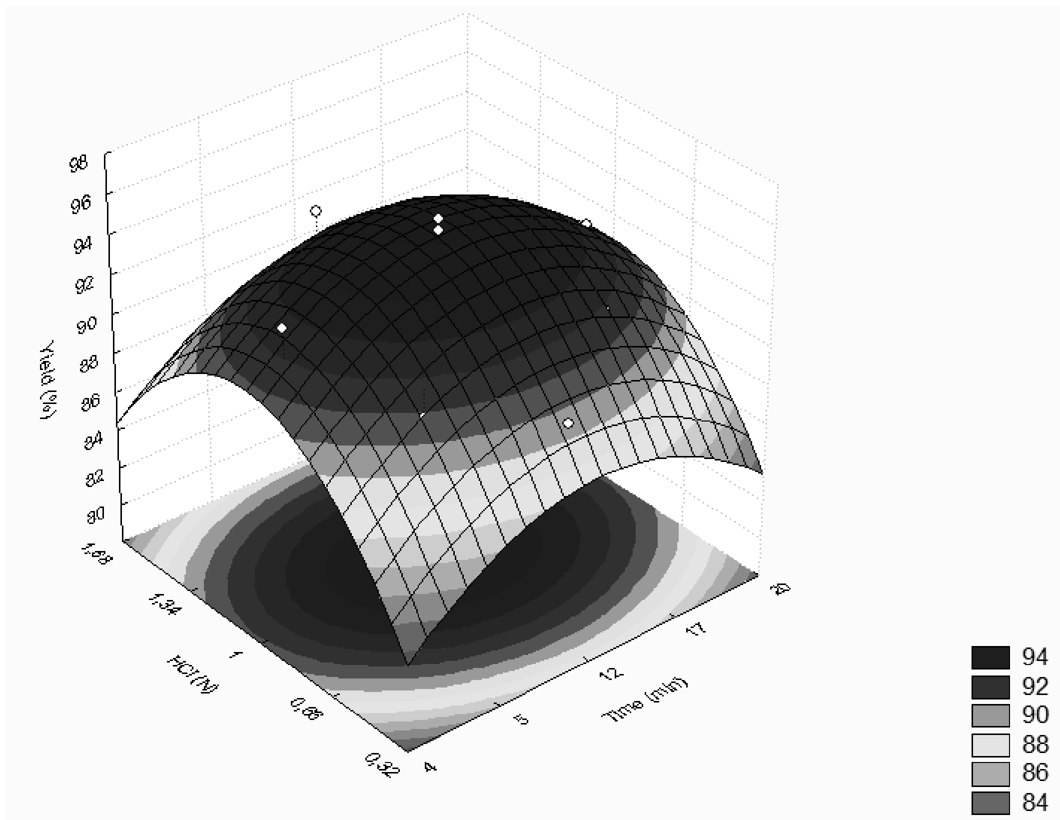

Figure 5- Response surface curve of hydrolysis yielding showing optimal range and values of HCl and Time.

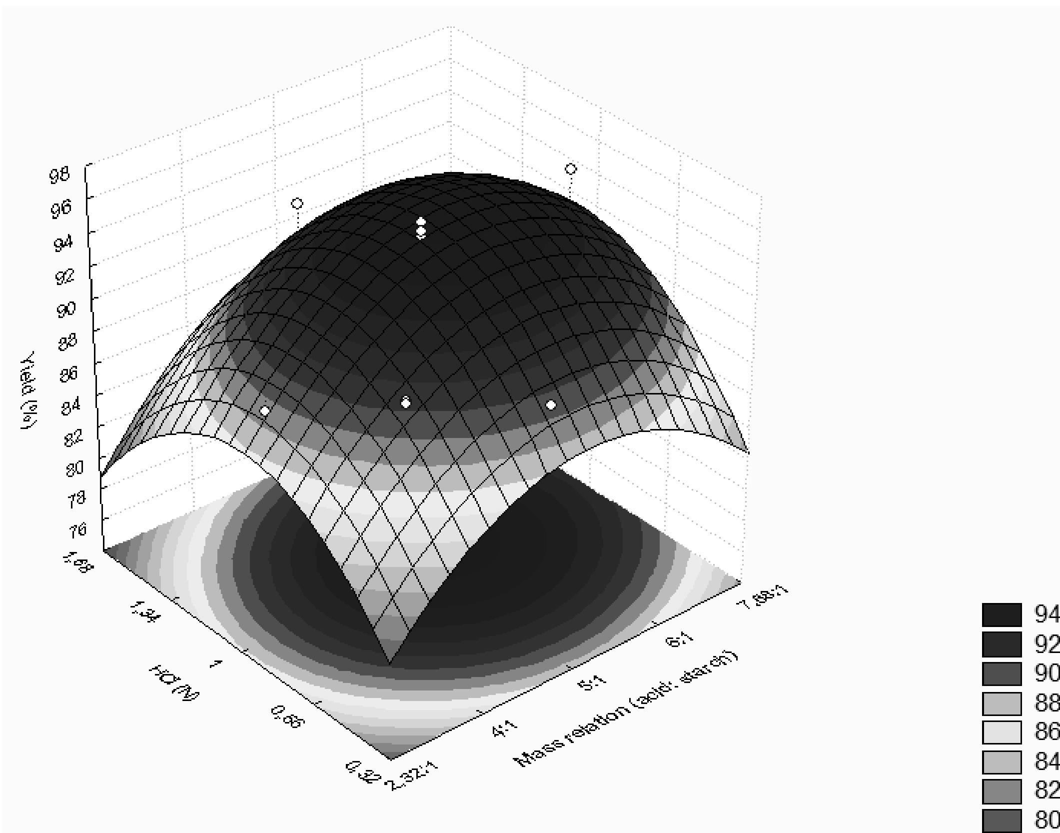

Figure 6- Response surface curve of hydrolysis yielding showing optimal range and values of HCl and Mass relation.

WOICIECHOWSKI *et al.* (2002) studying the acid and enzymatic hydrolysis of cassava bagasse for the recovery of reducing sugars and to establish the operational costs and they related optimal same conditions of hydrolysis regarding HCl concentration and time hydrolysis at 121°C. However, in this study, it was also investigated different mass content in operations. Based on the results obtained, it is possible to confirm despite the fact pressure conditions can be a scaling up process problem, it is possible to convert high amounts of starchy matter in short time.

3.4 Microscopy

After microscopic analysis, it can be observed in Figures 7 and 8, high amount of free cassava starch with its characteristic rounded shape. Besides the presence of a lot of free granules, the untreated material also has fibers with high amount of starch bonded, as previously observed by LEONEL, CEREDA and ROAU (1999).

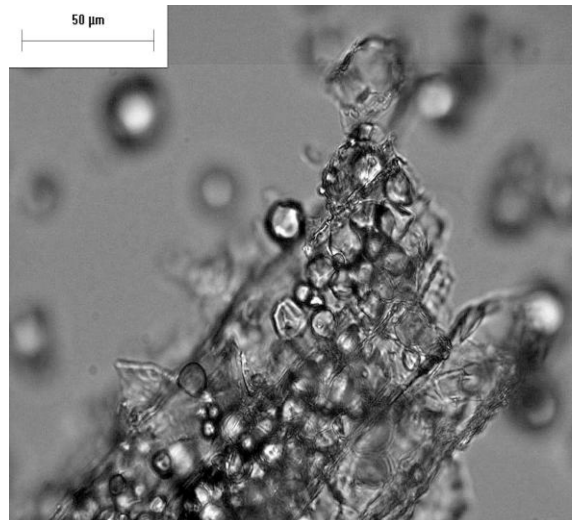

Figure 7 - Photomicrograph of cassava tuber flour 400X.

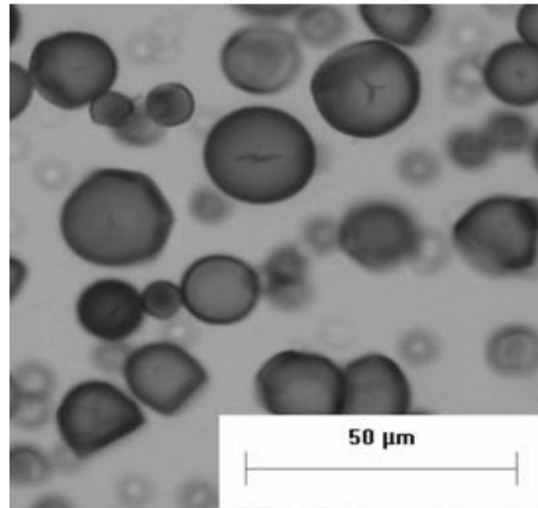

Figure 8 - Photomicrograph of cassava bagasse (Source: Lacerda *et al.*, 2009).

The average granule size of cassava starch was about 10 - 20µm. In other reports on cassava starch the granule size has been reported as 15 µm (SRIROTH *et al.*, 1999)

3.5 Thermal analysis

Results of Differential Scanning Calorimetry (DSC) are presented to determinate gelatinization parameters. Starch gelatinization is a process that breaks down the intermolecular bonds of starch in aqueous medium and under heat, allowing the hydrogen bonding sites (the hydroxyl hydrogen and oxygen) to engage more water. The gelatinization process is presented in the DSC as and endothermic event. Figure 9 represents energy variation of starch transformation as function of temperature increasing.

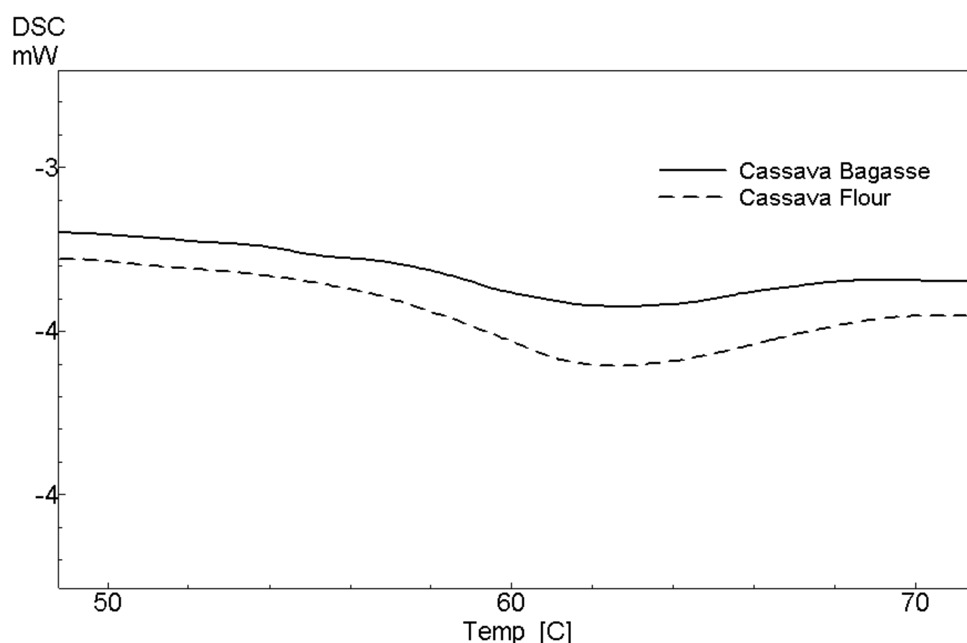

Figure 9 - Gelatinization curves of cassava bagasse and cassava tuber flour.

Many factors influence the gelatinization process, such as heating time, temperature and water content. Regarding Industrial applications, the knowledge of starch gelatinization can be useful. A low gelatinization temperature may provide energy savings in a large manufacturing operation. A narrow gelatinization range also will make production more efficient by making gelatinization quickly (TONGDANG; MEENUN; CHAINUI, 2008). Event peak at around 62°C (Table 5) regarding gelatinization of cassava starch was also previously achieved by other authors (TULYATHAN *et al.*, 2006).

Table 5- Gelatinization properties of cassava bagasse and cassava flour.

Biomass	Event on-set (°C)	Event peak (°C)
Cassava bagasse	57.32	62.67
Cassava tuber flour	56.84	62.70

Conventional plastics have large impact in increasing the environment's pollution. Thus, the interest has turned towards novel partially and completely biodegradable polymers (SCHLEMMER; SALLES, 2007). Structural resistance of starches is a very important factor on biodegradable plastics studies and the knowledge regards on blending of starch and polymers. DSC endothermic

profile curve, showed in Figure 10, is related to melting event of starch presented in studied matter. The enthalpy required for the event was estimated as showed in Table 6, confirming thermal event result observed previously by SRIROTH *et al.*, (2000).

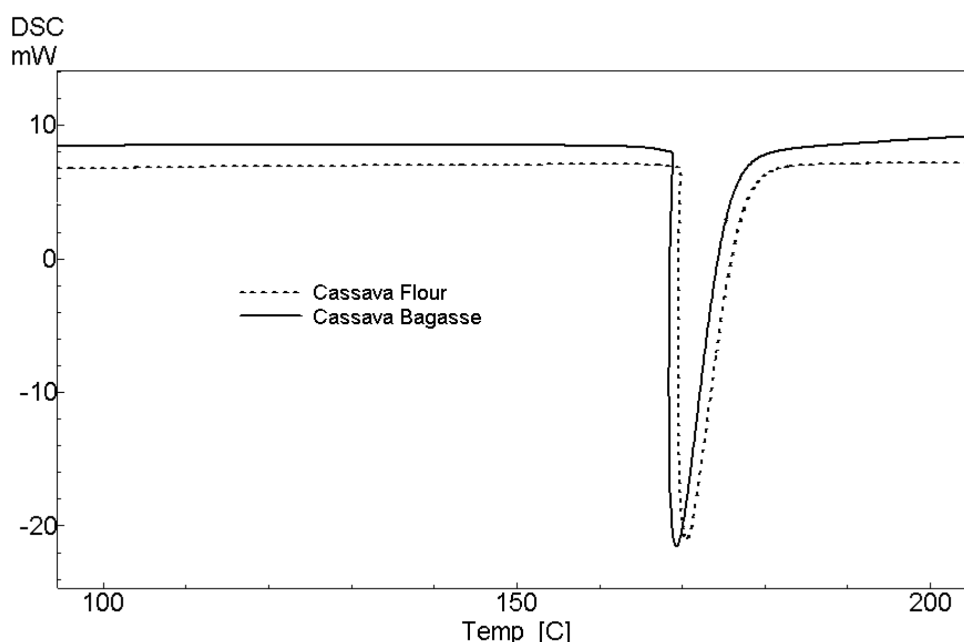

Figure 10- DSC curves related to starch melting of cassava bagasse and cassava tuber flour.

Table 6- DSC temperatures and energy related to starch melting of cassava bagasse and cassava tuber flour.

Biomass	Event on-set (°C)	Event peak (°C)	ΔH (J/g)
Cassava bagasse	169.23	170.93	132.55
Cassava tuber flour	169.79	170.54	136.31

Two stages of mass loss were observed and confirmed by DTG analyses (Fig. 11 and 12). Initial mass losses temperatures are presented in Table 7 and all analyzed samples showed three main mass losses events. As described by LEVAN (1998) in all TG curves observations, from 30°C to 150°C, mass loss regards to the elimination of moisture content; the second loss occurs between 200 and 400°C presents the decomposition of hemicellulose and cellulose the third loss stage above 400°C probably concerns to the decomposition of lignin and ash formation. Hemicellulose is less stable than the cellulose because its

side chains and degrades before cellulose. The lignin degrades throughout hemicellulose and cellulose degradation processes (SHAFIZADEH; DEGROOT, 1976).

Table 7- DTA, DTG and TG temperature values related to mass loss of cassava bagasse and cassava tuber flour.

Biomass	DTA		DTG		TG			
	Peak 1 (°C)	Peak 2 (°C)	Peak 1 (°C)	Peak 2 (°C)	On-set 1 (°C)	On-set 2 (°C)	Moisture (%)	Total loss (%)
Cassava bagasse	343.13	446.53	305.32	440.85	265.43	446.09	11.19	98.26
Cassava tuber flour	339.39	447.44	301.74	431.40	272.99	445.17	10.57	99.01

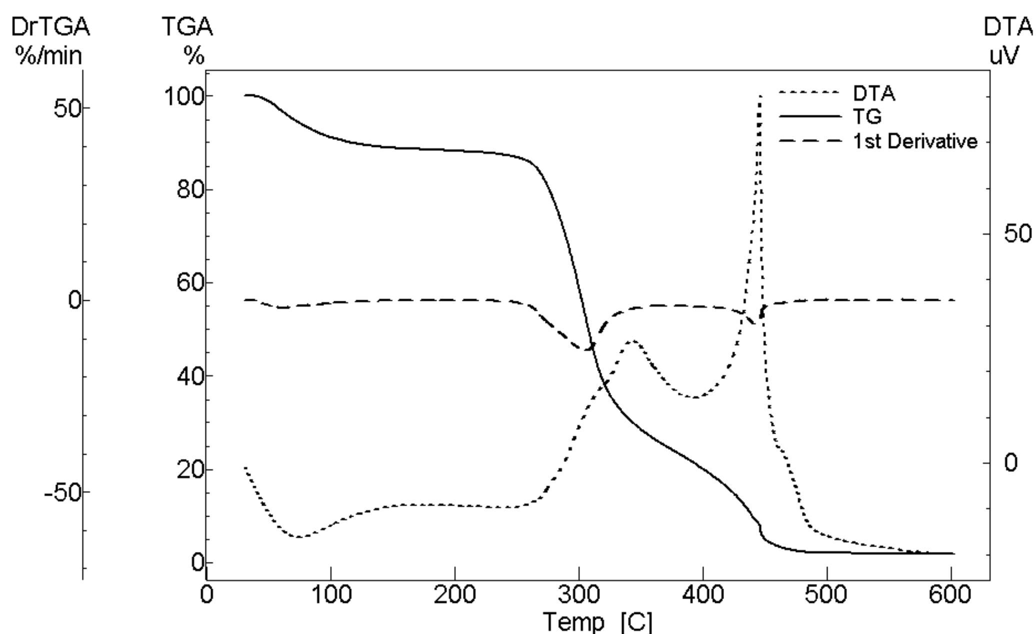

Figure 11- TG, DTA and DTG curves of cassava bagasse.

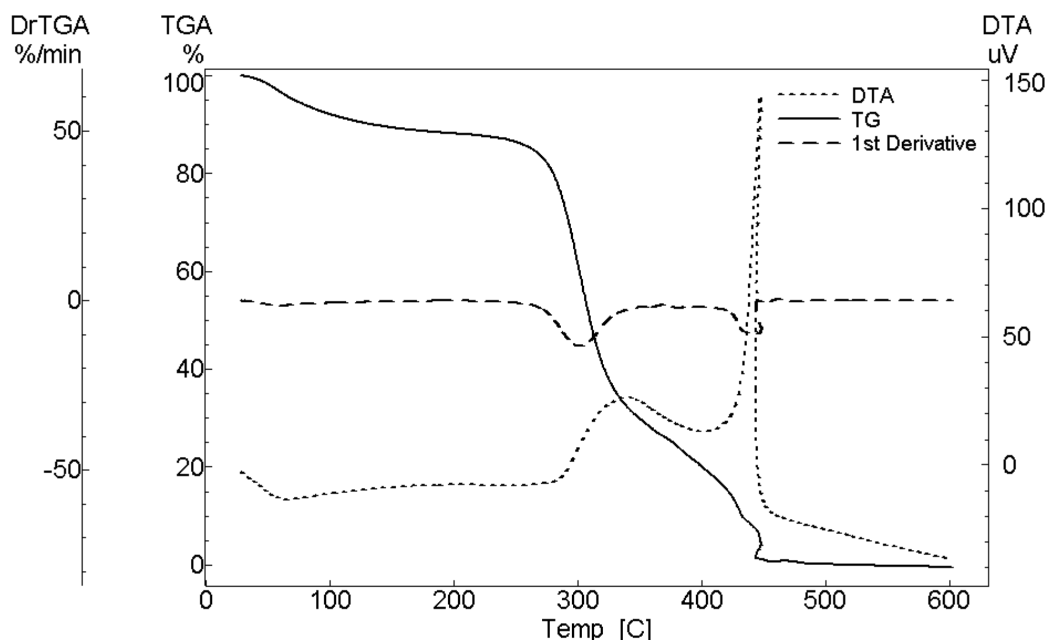

Figure 12 - TG, DTA and DTG curves of cassava tuber flour.

TG and DTA curves also suggest that the oxidation of the organic matter is accompanied by combustion. During the last step, the mass loss occurs through a slow process corresponding to the small and broad exotherm and a sharp exothermic peak around 446ºC is attributed to the total oxidation of the organic matter. BICUDO *et al.*(2009) observed the same behavior under synthetic air experiments studying the characterization of native starches of Paraná pine seeds (*Araucaria angustiofolia*, Bert O. Ktze) and European chestnut seeds (*Castanea sativa*, Mill).

4. CONCLUSION

Studied samples had variations regarding starch content among two methods. Probably acid hydrolysis condition is able to convert even starch present inside fibers. Microscopic studies assisted to understand that the enzyme acted initially on granules surface and especially in their irregularities. Thermogravimetry helped to observe starch hygroscopicity, thermal stability of raw state and how it decreased during hydrolysis due to the higher surface area. DSC had illustrated, with precision, a particular structural changing event of great importance for the industry: the starch gelatinization. Characterization

of raw material is a very important step regarding the knowledge of further application behaviors.

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CHAPTER III

YEAST STRAIN SCREENING AND MEDIUM OPTIMIZATION FOR PRODUCTION OF OLEAGINOUS BIOMASS BASED ON CASSAVA STARCH HYDROLYSATE.

ABSTRACT

This research was made to select a potential biomass/lipid producer from seven different yeast strains using glucose from cassava hydrolysate. After a first selection, where strain A (LPB0035) presented better results a Plackett Burman screening was applied to identify factors presence that influence the lipid production yielding positively and KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4$, $CaCl_2$, urea and yeast extract were determined. Finally, a 2^3-factorial central composite design (2^3-CCRD) was carried out in order to identify optimum parameter levels for the production based on significative factors observed in previous screening and following concentrations were established (g/L): KH_2PO_4 (0.5), $MgSO_4$(0.30), $CaCl_2$(0.30) and urea (1.77).

Keywords: cassava, Placket Burman, optimization, microbial lipid production.

1. INTRODUCTION

Recently, much attention has been given to development of microbial, oils and it has been found that many microorganisms, such as algae, yeast, bacteria, and fungi. Since they have the ability to accumulate oils under some special cultivation conditions, they have often been considered for the production of oils and fats as an alternative to agricultural and animal sources. Lipids serve as storage materials in some lipid accumulating microorganisms. It is reported that yeasts can store up to 70% of lipids in dry basis (GUERZONI *et al.*, 1985; KIMURA; YAMAOKA; KAMISAKA, 2006). First reported research concerning microorganism lipid accumulation had been reported already more than 40 years ago (MULDER *et al.*, 1962). These authors observed that under nitrogen limiting conditions and the presence of a carbon-source in excess organisms started to store lipids. It is been observed physical factors such as the concentration of some ions like Zn, Mn, Fe, Ca, K, and nitrogen sources affect lipid production (NAGANUMA *et al.*, 1985). The aim of the present work was to further investigate the potentiality of lipid production by several yeast strains, in broths having various initial C/N ratios and in the presence of some ions.

2. MATERIALS AND METHODS

2.1 Yeast maintenance and pre-cultures

In order to select a potential biomass/lipid producer, following yeast strains A (LPB 0035), B (LPB 0036), D (LPB 0040), H (LPB 0013), I (LPB 0038), J (LPB 0023) and N (LPB 0045) from Bioprocess and Biotechnology Laboratory (UFPR). All tested strains were maintained at 4 ºC on YM agar slant (20g/L agar, 0.3g/L of malt extract, 0.3g/L of yeast extract, 0.5g/L of peptone, 10g/L of glucose at pH 5.5) and sub-cultured twice a month. Inoculum were prepared by a 4 days culture on YM medium (0.3g/L of malt extract, 0.3g/L of yeast extract, 5g/L of peptone, 10g/L of glucose at pH 5.5) under agitation (120rpm) at 30°C. Then, pre-cultures were submitted to 168h cultivation at

120rpm, pH 5.5 (±0.5), 30°C under different nitrogen sources keeping C/N ratio about 32. Urea and yeast extract were used in a first moment and as a carbon source it was used hydrolyzed cassava tuber flour.

2.2 Plackett Burman screening method

Initial screening of the ingredients is done in order to understand the significance of their effect on the obtained results and then a few better ingredients are selected for further optimization (GREASHAM, 1983; NAVEENA *et al.*, 2005). Strain A was maintained and inoculum was prepared as described in initial screening. In order to select a composition media and to verify the importance on fermentation process the following nutrients were added based on previous studies: $MgSO_4 \cdot 7H_2O$, $CaCl_2$, KH_2PO_4, $ZnSO_4$, $FeSO_4$, urea, yeast extract, ammonium sulphate and glucose from previous cassava tuber hydrolysis. Pre-cultures were submitted to 168h cultivation at 120rpm, pH 5,5 (±0,5) at 30°C (DAI, C. *et al.*, 2007; HU, C. *et al.*, 2009). All experiments were carried out following Tables 1 and 2.

Table 1 – Plackett Burman screening experiments matrix.

	A	B	C	D	E	F	G	H	I	J	K
1	1	-1	1	-1	-1	-1	1	1	1	-1	1
2	1	1	-1	1	-1	-1	-1	1	1	1	-1
3	-1	1	1	-1	1	-1	-1	-1	1	1	1
4	1	-1	1	1	-1	1	-1	-1	-1	1	1
5	1	1	-1	1	1	-1	1	-1	-1	-1	1
6	1	1	1	-1	1	1	-1	1	-1	-1	-1
7	-1	1	1	1	-1	1	1	-1	1	-1	-1
8	-1	-1	1	1	1	-1	1	1	-1	1	-1
9	-1	-1	-1	1	1	1	-1	1	1	-1	1
10	1	-1	-1	-1	1	1	1	-1	1	1	-1
11	-1	1	-1	-1	-1	1	1	1	-1	1	1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

Table 2 – Nutrients used for Plackett Burman screening.

		Level:	
		-1	+1
A	KH_2PO_4	0	1.5g/L
B	$MgSO_4 .7H_2O$	0	1.5g/L
C	$FeSO_4$	0	0.1g/L
D	$CaCl_2$	0	0.5g/L
E	$ZnSO_4$	0	0.01g/L
F	Urea	0	0.5g/L
G	Yeast extract	0	1.5g/L
H	Amonium sulphate	0	1,0g/L
I	Glucose	40g/L	100g/L
J	Dummie*	0	0
K	Dummie*	0	0

2.3 Optimization using response surface methodology

A 2^3-factorial central composite design (2^3-CCRD) was carried out in order to identify optimum parameter levels for the production. The parameters (or independent variables) studied were: cassava bagasse (59.11%) and Integral cassava flour (90.57%). In this study we observed some points considered critical, as, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, CaCl2 and Urea in determining the Lipid Yielding, using a 2^4 full factorial experimental design with four independent variables. To estimate the experimental error, we used three replicates of the experiment corresponding to the central points. To measure the possibility of non-linearity in the concentration values for Lipid Yielding against the four factors outlined in this experiment, eight (8) axial points were added to the center of the complete experiment planning. The experimental design with values are presented in Table 3. The response (Y) or dependent variable was the concentration of Lipid Yielding (g/L). The first 16 experiments of Table 3 were sufficient for determining the linear model and refer to the complete experiment 2^4. Exeperiments 17 to 24 of planning are the axial points and three replicates of the experiment, which are the central points regarded as three last experiments. The distance of the axial points was ± 2,00, calculated from Equation 1:

$$\alpha=(2n)^{1/4} \qquad (1)$$

Where α is the distance of the axial points and n is the number of independent variables. Strain A pre-cultures (10% volume) were submitted to 500mL erlenmeyer flasks (total broth volume of 200mL) for 168h cultivation at 120rpm, pH 5.5 (±0.5) at 30°C. The experiments followed matrix as shown in Tables 3 and 4. All data were treated with STATISTICA® 5.0 from Statsoft Inc.

Table 3 – 2^4 full factorial experimental design experiments matrix.

Experiment	A	B	C	D
1	-1	-1	-1	-1
2	-1	-1	-1	1
3	-1	-1	1	-1
4	-1	-1	1	1
5	-1	1	-1	-1
6	-1	1	-1	1
7	-1	1	1	-1
8	-1	1	1	1
9	1	-1	-1	-1
10	1	-1	-1	1
11	1	-1	1	-1
12	1	-1	1	1
13	1	1	-1	-1
14	1	1	-1	1
15	1	1	1	-1
16	1	1	1	1
17	-2	0	0	0
18	2	0	0	0
19	0	-2	0	0
20	0	2	0	0
21	0	0	-2	0
22	0	0	2	0
23	0	0	0	-2
24	0	0	0	2
25 (C)	0	0	0	0
26 (C)	0	0	0	0
27 (C)	0	0	0	0

Table 4 – 2^4 full factorial experimental design variables and levels.

		Level:				
		-2	-1	0	+1	+2
A	KH_2PO_4	0.25	0.50	0.75	1.00	1.25
B	$MgSO_4.7H_2O$	0.15	0.30	0.45	0.60	0.75
C	$CaCl_2$	0.15	0.30	0.45	0.60	0.75
D	Urea	1.27	1.77	2.15	2.59	3.43

2.4 Determination of yeast dry weight

Yeast dry weight (biomass dry weight) determination was performed by collecting 35mL from harvesting broth. Wet cells were obtained by centrifugation at 5700 rpm, during 15 min. and cell dry weight was obtained by drying at 100°C to constant weight. The supernatant was collected and freezed to further DNS analyses.

2.5 Reducing sugars determination

The supernatant collected during determination of yeast dry weight process, was analysed by DNS method (MILLER, 1959) using methodology described by LACERDA *et al.* (2009) and was expressed as gram per liter.

2.6 Biomass lipid concentration

Lipid content produced by cultivations were estimated from dry biomass following Folch *et al.* (1957) method.

3. RESULTS AND DISCUSSION

3.1 Strain screening

Tables 5 and 6 show results obtained from initial screening using several yeast strains under same condition. It can be observed strain A (*Rhodosporidium toruloides* LPB0035) provided best results regarding biomass and lipid accumulation. *R. toruloides* strains are potentially lipid producers under special conditions as reported by several researches (EVANS; RATLEDGE, 1984; LI et al, 2006; LI; DU; LIU, 2008; LIU et al, 2009; ZHAO et al, 2010).

Table 5 – Initial screening using yeast extract as nitrogen source.

Strain	Biomass g/L	% Lipids	Final reducing sugars	Final pH	Lipid Yielding(g/L)
A	9.77	17.11	32.90	5.25 (±0.03)	1.67 (±0.13)
B	3.41	9.91	60.83	4.05 (±0.01)	0.33(±0.18)
D	1.71	24.49	63.26	5.05 (±0.00)	0.41 (±0.01)
H	2.19	15.56	55.37	4.21 (±0.11)	0.34 (±0.17)
I	2.83	24.68	39.04	4.24 (±0.21)	0.69 (±0.03)
J	4.95	17.43	45.30	4.32 (±0.10)	0.86 (±0.13)
N	5.61	17.09	40.01	5.21 (±0.13)	0.96 (±0.10)

Table 6 – Initial screening using urea as nitrogen source.

Strain	Biomass g/L	% Lipids	Final reducing sugars	Final pH	Lipid Yielding(g/L)
A	7.37	38.52	19.83	5.45 (±0.02)	2.83 (±0.07)
B	4.33	29.48	38.29	4.95 (±0.04)	1.27 (±0.02)
D	2.12	32.07	60.21	5.25 (±0.02)	0.67(±0.15)
H	4.01	31.5	52.60	5.34 (±0.03)	1.26 (±0.18)
I	2.96	28.98	45.27	5.67 (±0.11)	0.85 (±0.20)
J	6.66	27.21	22.18	5.47 (±0.03)	1.81 (±0.09)
N	6.12	28.26	45.67	5.51 (±0.10)	1.72 (±0.16)

It is noted when using urea as nitrogen source there is higher yielding in lipids accumulation. Xue *et al.* (2006) comparing four yeast strains to produce lipid rich biomass, observed the best results for biomass production, lipid accumulation and chemical oxygen demand degradation after 5 day culture with urea as nitrogen source instead of only yeast extract. In addition, residual reducing sugars analyses demonstrated strain “A” provided highest conversion during cultivation using urea and yeast extract. Based on those previous results, strain “A” was used for further studies presented in this research.

3.2 Plackett Burman Screening

A well done statistical method offers several advantages over conventional method being feasible and reliable, short lists significant nutrients, helps understanding the interactions among them at different concentrations and can reduce the number of experiments tremendously resulting in saving time, glassware, chemicals and manpower (SRINIVAS *et al.*, 1994; CARVALHO *et al.*, 1997). Before building a factorial planning, a screening design as Plackett Burmann can be an important tool to define variables influence significatively in lipid production. The screening design produced results showed in Table 7.

Table 7- Plackett Burman matrix results.

	Biomass g/L	% Lipids	Reducing Sugars Conversion (%)	Final pH	**Lipid Yielding (g/L)**
1	9.64	24.97	10.85	4.5	**2.41**
2	9.10	24.52	14.18	4.16	**2.06**
3	7.41	36.39	20.26	5.01	**2.19**
4	9.35	26.54	32.45	5.12	**2.36**
5	9.75	36.08	49.33	5.19	**2.99**
6	8.98	20.68	45.46	4.15	**1.86**
7	10.94	20.57	18.05	4.35	**2.25**
8	8.78	15.8	25.52	4.14	**1.42**
9	8.85	30.31	45.73	4.25	**2.68**
10	9.79	20.44	15,83	4.79	**1.97**
11	10.00	25.99	37.16	4.52	**2.59**
12	7.05	39.55	43.25	4.85	**2.86**

From the results in Table 8 is possible to confirm with 95% confidence that the variables A, B, D, F and G will positively influence the results and together account for 99.51% of the explanatory power of these variables in the biomass gain.

Table 8 – Estimation by point, by interval and hyphothesis tests to the effects.

	Effect	Std.Err.	t(2)	p	-95,% Cnf.Limt	+95,% Cnf.Limt	Coeff.	Std.Err. Coeff.	-95,% Cnf.Limt	+95,% Cnf.Limt
Mean/Interc.	9.137	0.051	180.491	0.000	8.919	9.354	9.137	0.051	8.919	9.354
(1)A	**0.597**	**0.101**	**5.893**	**0.028**	**0.161**	**1.032**	**0.298**	**0.051**	**0.081**	**0.516**
(2)B	**0.453**	**0.101**	**4.478**	**0.046**	**0.018**	**0.889**	**0.227**	**0.051**	**0.009**	**0.444**
(3)C	0.093	0.101	0.922	0.454	-0.342	0.529	0.047	0.051	-0.171	0.264
(4)D	**0.650**	**0.101**	**6.420**	**0.023**	**0.214**	**1.086**	**0.325**	**0.051**	**0.107**	**0.543**
(5)E	-0.420	0.101	-4.148	0.053	-0.856	0.016	-0.210	0.051	-0.428	0.008
(6)F	**1.030**	**0.101**	**10.174**	**0.010**	**0.594**	**1.466**	**0.515**	**0.051**	**0.297**	**0.733**
(7)G	**1.360**	**0.101**	**13.433**	**0.005**	**0.924**	**1.796**	**0.680**	**0.051**	**0.462**	**0.898**
(8)H	0.177	0.101	1.745	0.223	-0.259	0.612	0.088	0.051	-0.129	0.306
(9)I	0.303	0.101	2.996	0.096	-0.132	0.739	0.152	0.051	-0.066	0.369

Effect Estimates; Var.:RESULT; R-sqr=,99515; Adj:,9733

The percentage of lipids not statistically corresponds to the amount of biomass formed (p> 0.05). It is possible the combination of salts and nitrogen sources did not affect the relation of production among lipids and biomass during fermentation. The same situation occurs in the evaluation of performance in the lipid end of fermentation. In this case, the assessment by Plackett-

Burman shows that the variables A, B, D and G are factors that influence the outcome positively, but the claim has no statistical significance (p> 0,05) probably due to the fact that there is proportionality between the formation of biomass and the formation of lipids in the same experiment.

3.3 Media optimization

Media optimization using 2^4 full factorial experimental design resulted in table 9 where it is possble to observe central point provided best results in lipid yielding and reducing sugars consumption.

Table 9 – Matrix showing 2^4 full factorial results of experiments.

Trial	Biomass (g/L)	% Lipids	Reducing Sugars Conversion (%)	Final pH	**Lipid Yielding (g/L)**
1	8.650	24.24	23.27	4.9	2.10
2	9.980	26.16	38.67	5.6	2.61
3	8.970	20.15	37.71	6.0	1.81
4	9.870	20.63	45.55	6.1	2.04
5	9.710	28.32	35.37	6.0	2.75
6	9.100	30.01	24.65	5.5	2.73
7	10.960	34.21	25.33	5.6	3.75
8	9.200	33.57	37.02	6.1	3.09
9	9.030	31.83	26.71	6.8	2.87
10	10.450	30.14	21.08	5.7	3.15
11	11.170	25.06	38.40	6.5	2.80
12	10.340	21.45	15.02	5.7	2.22
13	11.120	14.28	26.03	5.8	1.59
14	9.290	19.45	13.92	5.1	1.81
15	11.280	14.99	9.93	5.7	1.69
16	9.990	15.67	15.71	5.0	1.57
17	8.670	23.28	20.11	6.1	2.02
18	10.680	21.54	33.31	5.7	2.30
19	9.460	23.7	24.37	5.7	2.24
20	10.110	21.45	14.61	5.5	2.17
21	9.500	23.12	34.27	5.9	2.20
22	10.280	19.14	14.61	5.8	1.97
23	9.030	19.00	35.23	5.7	1.72
24	9.090	19.46	34.27	6.2	1.77
25	11.560	34.78	42.93	5.4	**4.02**
26	10.650	35.01	42.15	5.1	3.73
27	10.490	33.15	42.91	5.5	3.48

It is known nutrient imbalance in the culture medium triggers lipid accumulation in oleaginous microorganisms. When cells run out of a key nutrient, normally nitrogen, they cannot multiply and excess carbon substrate is assimilated continuously to produce storage lipids. Therefore, initial C/N ratio is very important for lipid accumulation (YONGHONG et al, 2006). Thus several initial C/N ratios were studied by fixing initial glucose concentration and central point combination presented best results regarding overall lipid yielding and best results were observed when the C/N ratio was 36.32.

There was verified in this study that the effects provided in Table 10 are statistically significant as shown by the values of (p) and (t) student for lipid yielding, although the factors of greatest significance for the model were quadratic effects of each factor. As these values were increased, these factors contributed negatively to the response (Y) model.

Table 10 – Estimation by point, by interval (95%) and hyphothesis tests to the effects.

Mean/Interc.	Regressn Coeff .	Std.Err.	t(12)	p	-95,% Cnf.Limt	+95,% Cnf.Limt
	3,743333	,211875	17,66761	,000000	3,281696	4,204970
(1)VAR1 (L)	-,109167	,074909	-1,45732	,170695	-,272380	,054047
VAR1 (Q)	-,335417	,079453	-4,22156	,001186	-,508531	-,162303
(2)NEWVAR2 (L)	-,031667	,074909	-,42273	,679966	-,194880	,131547
NEWVAR2 (Q)	-,324167	,079453	- 4,07997	,001526	-,497281	-,151053
(3)NEWVAR3 (L)	-,045833	,074909	-,61185	,552053	-,209047	,117380
NEWVAR3 (Q)	-,354167	,079453	-4,45755	,000782	-,527281	-,181053
(4)VAR2 (L)	-,001667	,074909	-,02225	,982615	-,164880	,161547
VAR2 (Q)	-,439167	,079453	-5,52736	,000130	-,612281	-,266053
1L by 2L	-,508750	,091745	-5,54528	,000127	-,708645	-,308855
1L by 3L	-,102500	,091745	-1,11723	,285766	-,302395	,097395
1L by 4L	-,016250	,091745	-,17712	,862367	-,216145	,183645
2L by 3L	,192500	,091745	2,09821	,057728	-,007395	,392395
2L by 4L	-,063750	,091745	-,69486	,500375	-,263645	,136145
3L by 4L	-,132500	,091745	-1,44422	,174275	-,332395	,067395

Regr. Coefficients; Var.:VAR3; R-sqr=,87681; Adj:,7331; 4 factors, 1 Blocks, 27 Runs;
MS Residual=,1346736; DV: VAR3

The interaction among $MgSO_4 \cdot 7H_2O$ and $CaCl_2$ resulted in a linear positive effect for response (Y). It was observed negative effects regarded as combination of KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ contribute in a minor scale for an absolute decreasing of lipid yielding. Equation 2 represents experimental modelo of lipid yielding as function of studied factors.

$$y=3.74-0.3354x_1^2-0.324x_2^2 -0.439\ x_3^2 +0.192\ x_2x_3 \qquad (2)$$

In this study during lipid yielding determinaton process, variables KH_2PO_4 (g/L) and $MgSO_4 \cdot 7H_2O$ showed a wide response as can be observed in Figure 1. Maximum levels for the determination of lipids yielding were found in an interval variation around central point for $MgSO_4 \cdot 7H_2O$ (0.75 g/L) and high concentration of $MgSO_4 \cdot 7H_2O$ (1.25 g/L). Figure 2 shows experiments carried out in central points produced best results when interaction of KH_2PO_4 (0.75g/L) and $CaCl_2$ (0.45g/L). Response surface curve showed in Figure 3 regards lipid yielding optimum regions for urea and $MgSO_4.7H_2O$ close to central points. The same behavior can be observed in Figures 4 and 5.

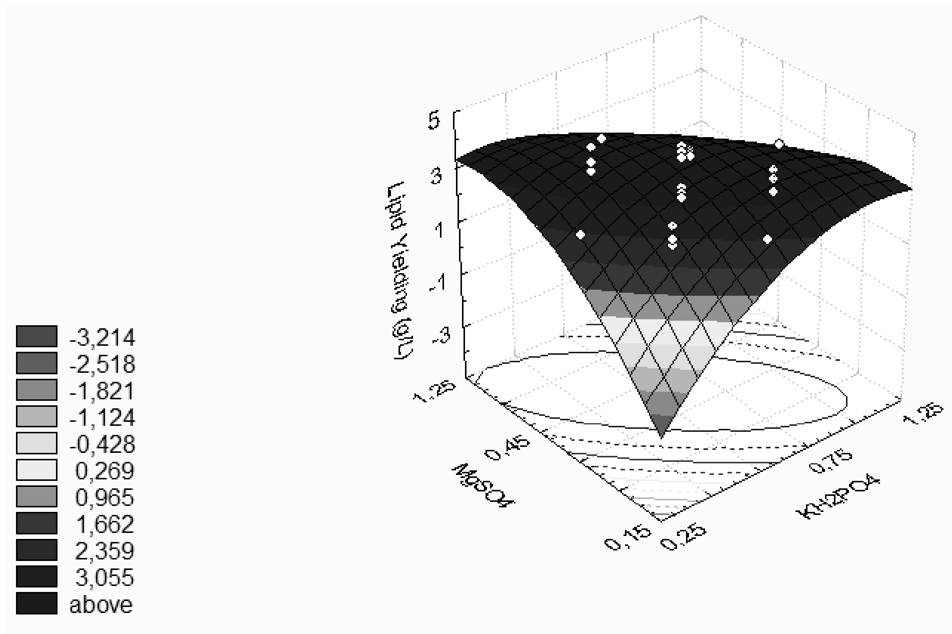

Figure 1 – Response surface curve of lipid yielding showing optimum regions of $MgSO_4 . 7H_2O$ and KH_2PO_4.

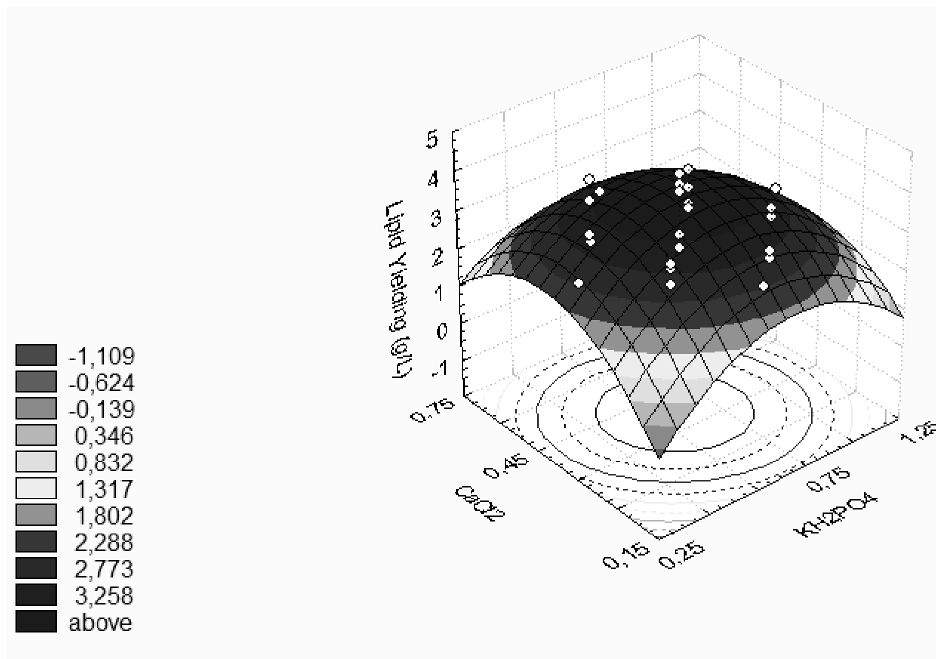

Figure 2 – Response surface curve of lipid yielding showing optimum regions of KH_2PO_4 and $CaCl_2$.

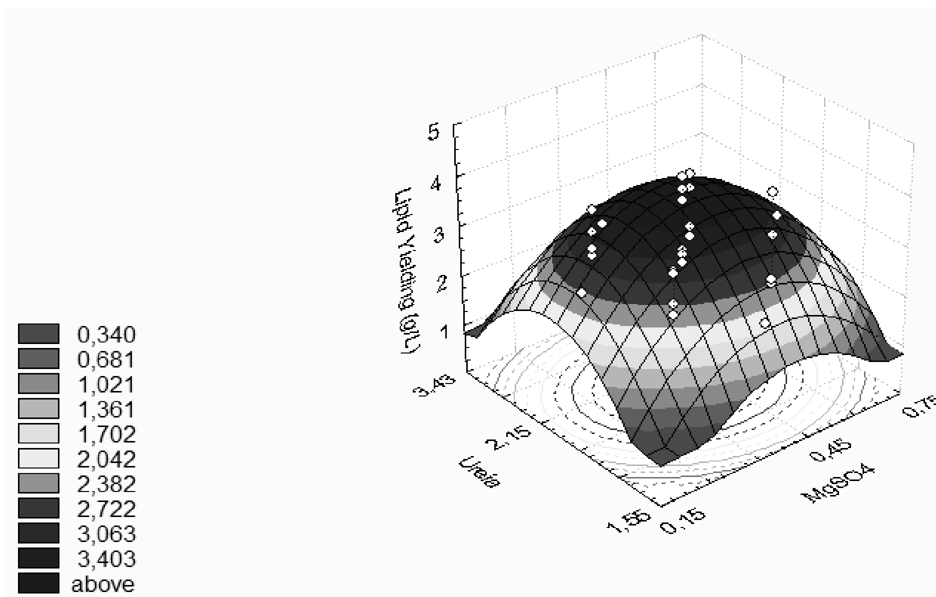

Figure 3 – Response surface curve of lipid yielding showing optimum regions of Urea and $MgSO_4.7H_2O$.

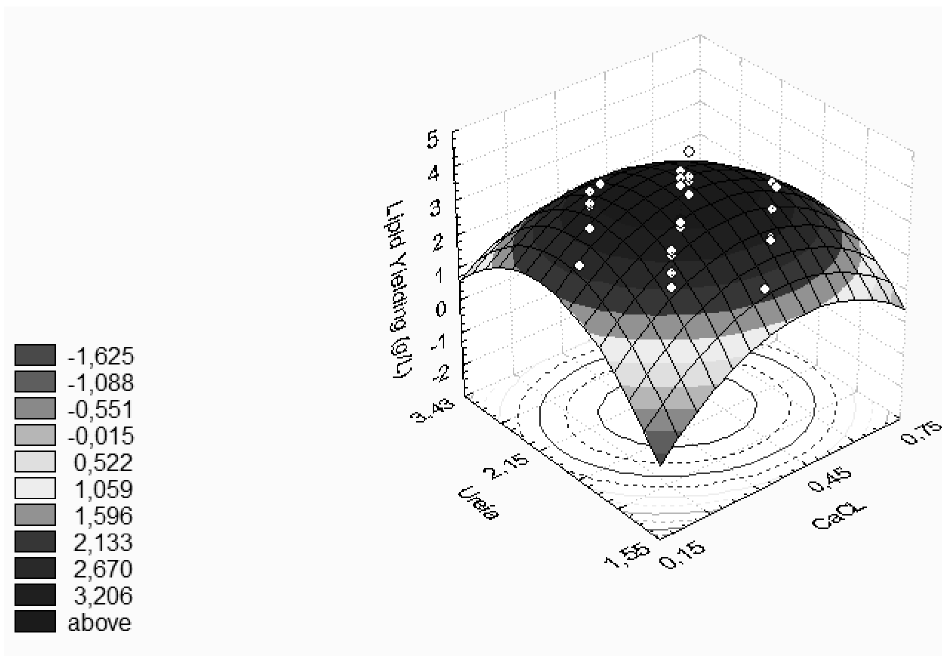

Figure 4 – Response surface curve of lipid yielding showing optimum regions of Urea and $CaCl_2$.

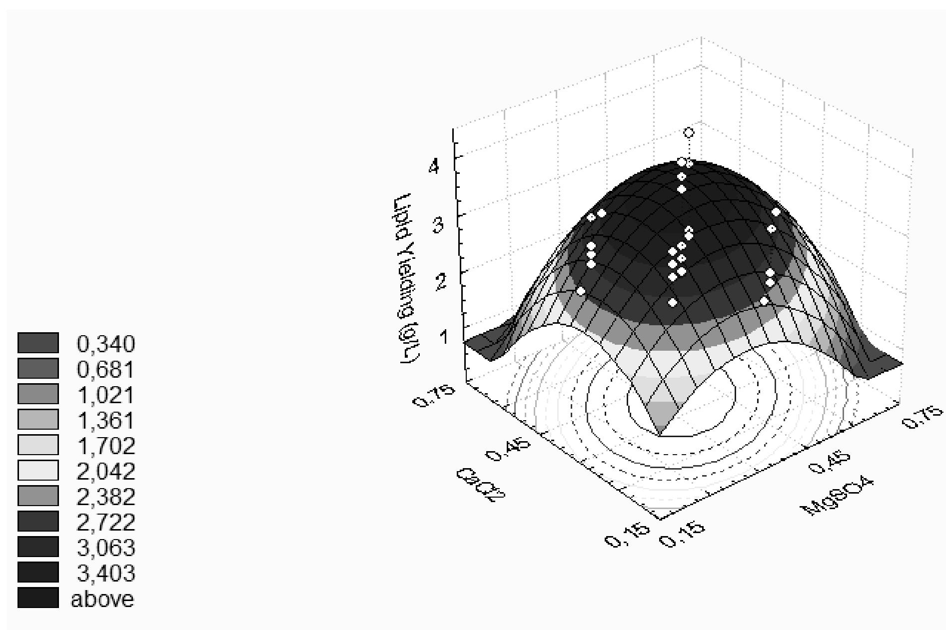

Figure 5– Response surface curve of lipid yielding showing optimum regions of $CaCl_2$ and $MgSO_4.7H_2O$

After response surface analysis is possible do observe optimum results for lipid yielding was established as (g/L): KH_2PO_4 (0.5-1.0), $MgSO_4$• $7H_2O$ (0.30- 0.60), $CaCl_2$(0.30-0.60). It is importantto note KH_2PO_4 and $CaCl_2$ were most important factors regarding responses and values close to central point

(0.30-0.60 g/L) benefits the lipid yielding. Urea addition did not present significane to lipid yielding , then minor value (1.77 g/L) was established.

4. CONCLUSION

In order to achieve a high-density cell culture for microbial lipid fermentation, different substrates and cultivation modes have been widely studied (LI; ZHAO; BAI, 2007). The results from flask batch cultures probably cannot provide optimal results regarding microbial biomass evolution due to many limitant factors like dissolved oxigen (dO_2), temperature and pH control. However, small scale trials can help to provide important information on broth composition and enable several conditions at the same time. Thus, scalling up processes can have some optimized initial conditions.

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CHAPTER IV

FED BATCH PROCESS FOR LIPID RICH BIOMASS PRODUCTION (*R. TORULOIDES* LPB 0035) USING HYDROLYZED STARCH AS CARBON SOURCE IN ORDER TO PRODUCE BIODIESEL.

ABSTRACT:

In this study, after testing process parameters, we found that *R. toruloides* LPB 0035 could accumulate up to 56.7% (w/w) oil from hydrolysate of cassava tuber and its cell dry weight reached 44.3 g/L at the end of fed batch cultivation. It was observed at the end of best results cultivation trial, 16.33% of reducing sugar remained in the fermented broth. Effluent from batch fermentation presented High biochemical demand oxygen (BOD) and chemical oxygen demand (COD) values. This liquid residue was submitted to microalgae cultivation in order to reduce BOD, COD concentrations, to produce oleaginous biomass suitable for biodiesel production and to use CO_2 previously produced. After 17 days of cultivation, *C. vulgaris* LPB 0033 the final broth achieved more than 50% BOD and COD reductions. Most of the fatty acids from the yeast and microalgae cultivation were C16:0, C18:1 and C18:2. Biodiesel produced using studied process present suitable characteristics for official international biodiesel specifications.

Keywords: Biodiesel, yeast, cassava, microalgae, integrated bioprocess.

1. INTRODUCTION

As fossil resources are diminishing, it has been realized that sustainable development requires fuels and chemicals to be produced from renewable resources, such as biomass derived carbohydrates. In this context, microbial lipids have attracted much attention in recent years. The efficiency of oil biosynthesis and its composition obtained from by yeast or other microorganism depend on the genetic properties of the strains, cultivation conditions and the composition of culture medium (EL-FADALY; EL-NAGGAR; MARWAN, 2009).

The production cost is one of the major factors limiting a broader use of microbial lipids. Although microbial lipids are currently less feasible than vegetable oil, methods are potentially valuable to improve the techno-economics of lipid production processes. The use of residues (i. e. cassava bagasse, lignocellulosic biomasses and sewage) and alternative crops (i. e. jersualem artichoke) as the feedstock has been studied and their use can be the key for the process scaling up feasibility (ZHAO, 2005; ANGERBAUER et al, 2008; ZHAO *et al.*, 2010). Microbial oils, produced by various microorganisms, are recognized as a potential source for biodiesel production due to their characteristics such as they are not affected neither by seasons nor by climates, they can accumulate high lipid content, can be produced from a wide variety of sources with short period of production, especially from the residues with abundant nutrition, and others (XUE *et al.*; 2006). The red yeast *Rhodosporidium toruloides* is a known microbial lipid producer and the characterization the oleaginous profile of *R. toruloides* CBS 14 has been reported (EVANS; RATLEDGE, 1984). During last years, microalgae cultivations have been extensively studied since it use CO_2 efficiently because they can grow rapidly and can be readily incorporated into engineered systems, such as photobioreactors (CHIU, 2008). This research aimed the production of biodiesel from *R. toruloides* LPB0035 using starchy matter hydrolysate as carbon source and use waste water from fed batch process in *C. vulgaris* LPB0033 cultivation.

2. MATERIALS AND METHODS

Chemicals and reagents were bought locally and were of analytical reagent grade. *R. toruloides* LPB 0035 was maintained at 4 ºC on YM agar slant (0.3% malt extract, 0, 0.3% yeast extract, 5% peptone, 2% agar) and sub-cultured twice a month. First initial culture was prepared by a 72h culture on 100 mL of YM medium (0.3% malt extract, 0.3% yeast extract, 5% peptone, pH 5.5) under agitation (120rpm) at 30 ◦C in 500mL erlenmeyer flasks.

2.1 Carbon source used

Glucose was the carbon source for fed batch process was obtained from three forms of starchy matter: In natura cassava roots, cassava bagasse and cassava integral flour. *In natura* cassava (*Manihot esculenta*) roots containing 61% moisture and 33% starch in wet basis were washed pelled, grinded and a mass based mixture of 1:1 (cassava:1.34Mol/L HCl) was prepared sealed with aluminum foil and rubber band. Cassava integral flour containing 10% moisture and 81% starch in wet basis was also used and mass based mixture of 1:5 (cassava:1.0 Mol/L HCl) was prepared sealed with aluminum foil and rubber band. Both flasks were submitted to a acid hydrolysis process during 12 min. at 121°C. After hydrolysis, pH was adjusted to 3,5 using NaOH 1,0Mol/L, filtered through cotton filter and submitted to centrifugation at 4700 rpm. Fed solution was obtained by concentrating glucose up to 500g/L using a rotatory evaporator equipment under reduced pressure condition. The glucose solutions obtained was stored at 4°C.

2.2 Fed batch culture

The inoculum contained 1,1L of medium prepared by a glucose (40g/L), KH_2PO_4 (0.50g/L), $MgSO_4.7H_2O$ (0.30g/L), $CaCl_2$ (0.30g/L) and Urea (1.77g/L) broth was and inoculated with 60 mL of first initial cell culture on YM described before. The culture was incubated in an orbital shaker at a rotary rate of 120 rpm at 30 °C during 72h. The fed batch were performed in a 14L BioFlo 110 bioreactor filled with 7.9L of a glucose (75g/L), KH_2PO_4 (0.50g/L), $MgSO_4.7H_2O$

(0.30g/L), $CaCl_2$ (0.30g/L) and urea or yeast extract were added as nitrogen source in order to keep C/N=36.32 as described below. After heat sterilization during 30min. at 121ºC, the bioreactor was flushed with O_2 gas at 9vvm. The temperature was controlled at 30ºC, the agitation rate was setted to 250rpm (altered as function of dO_2 setting) and the pH was maintained at 5.6. Samples were collected in the beginning of the fermentation and at periodical intervals. Experimental tests were made as described in table 1.

Table 1 – Biorreactor fed batches experiments.

Experiment	Nitrogen source (g/L)	dO_2 (%)
1	Yeast extract: 7,52	30
2	Urea: 1,77	30
3	Yeast extract: 7,52	50
4	Urea: 1,77	50

2.3 Determination of yeast dry weight

Yeast dry weight (biomass dry weight) determination was performed by collecting 35mL from harvesting broth. Wet cells were obtained by centrifugation at 5700 rpm, during 15 min. and cell dry weight was obtained by drying at 100°C to constant weight. The supernatant was collected and feezed to further DNS analyses.

2.4 Reducing sugars determination

The supernatant collected during determination of yeast dry weight process, was analysed by DNS method (MILLER, 1959) using methodology described by Lacerda *et al.* (2009) and was expressed as gram per liter.

2.5 Oil recovery

Oil recovering from final wet biomass was made by broth centrifugation in buckets of 500 ml capacity at 4700 rpm for 15 minutes. Biomass recovered was

washed with distilled water and hydrolyzed with analytical grade HCl at 1kg wet biomass: 30g HCl. The mixture was kept warm in a exhaustion chapel at 70 ° C for 4 hours. The biomass hydrolyzate is then subjected to further centrifugation for 15 minutes at 5000 rpm and the free oil recovered by filtration. Then, residual biomass was kept in na onven at 70 ° C for 48 hours and free remaining oil was recovered through a funnel into a bottle. The remaining lipid fraction of biomass was extracted with hexane in Soxhlet condenser with a process in which the organic solvent dragged lipidic matter. The solvent is then recovered almost entirely through rotoevaporation under reduced pressure. The residual solvent was evaporated under heating at 70° C for 24 hours in an oven. A fraction of recovered oil was bleached and degummed with water at 50°C under continous agitation. Remained oil was kept in a flask to further analysis and transesterification. The productivity of oil produced (conversion coefficient) was also calculated according to the following equation:

$$\text{Single cell oil (lipids) production} = \frac{\text{Cell dry weight (g/L)}}{\text{Lipids content (\%)}}$$

After fatty acids methylation process described by METCALFE, SMITH and PELKA (1966), samples were submitted to detection and components separation by chromatography (AOCS, 1998). Gas cromatographic analysis was performed by using Varian (model 3300) equipped with flame detection and gun-type "split"/Splitless" under following conditions: Carbowax 20M column, detector temperature of 270°C and injector 250°C. The schedule was determined as follows: initial temperature = 165 ° C (15 min.) And increase by 5°C/min. until a final temperature = 200°C (6 min.). Ultrapure hydrogen carrier gas, flow rate 1.2 mL/min. to 30psi. Physical chemical parameters of obtained oil were determined following American Oil Chemists Society (2007) methods.

2.6 Oil Transesterification

Following (MARCHETTI, MIGUEL & ERRAZU, 2007), methyl and ethyl esters were produced in 1 liter bottles with aluminum by alkaline transesterification of the oil obtained from the microbial biomass of *R. toruloides*

LPB 0035 with methanol or ethanol in stoichiometric excess, using potassium hydroxide (KOH) as catalyst for the reaction in basic medium, in a molar ratio alcohol:oil 6:1 using 0.5% (w/w) catalyst at a reaction temperature of 65 (± 2)°C for 45 minutes under constant magnetic stirring. The fraction of residual alcohol was eliminated from the biodiesel, heating the product by 85°C for 15 min. After this period, the reaction product was transferred to a separatory funnel. The top fraction (biodiesel) was separated and washed by shaking with water temperature 50ºC and water was removed from separatory funnel.

2.7 Biodiesel analyses

Biodiesel was submitted to physical-chemical analyses following Official ASTM and EN Standards.

2.8 Microalgae cultivation

In this method adapted from SIDNEY et al (2010), *C. vulgaris* LPB 0033 strain was cultivated at 30 ºC for 17 days in 2L Erlenmeyers flasks The flasks were filled with 1.2 L of centrifuged broth from the production of oleaginous yeast inoculated with 10% v / v of an active culture microalgae Chlorella vulgaris OF so that the concentration at the beginning of cultivation is around 0.2 g /L. Agitation and aeration were provided by cpntinous injection of 1v/v/m (gas volume/medium volume/minute) of air enriched with 5% of CO_2 through porous stones. Illumination of culture was provided by eight cool white 32W fluorescent lamps (providing 3500 lux) in 12:12 h (light/dark) photoperiod. Samples were withdrawn daily and centrifuged in a Sorvall Legend Mach 1.6 R centrifuge (Sorvall, Germany) at 16000g for 15 min. Cells were washed once and dried at 60 ºC until constant weight.

2.9 Ion profile composition

Cations and anions compositions of broths before and after microalgae cultivation were determinated following SIDNEY et al (2010) using a 761 Compact IC 817 Bioscan chromatograph. For cation determination, the column

used was METROSEP C3 250/4.0 (Metrohm), 250 mL x 4.0 mm ID. Analytical conditions were as follows: 3.5 mM HNO_3, 1.0 mL/min, 40ºC, 20 µL of sample volume and 11.2 MPa. A standard chromatogram was prepared with the following salts: $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, KCl, Na_2SO_4, $ZnSO_4.7H_2O$ and NH_4Cl. All reagents used were of analytical grade (Sigma–Aldrich). For anions, the column used was a METROSEP A Supp 5 250/4.0 (Metrohm), 250mL x 4.0mmID. Analytical conditions were as follows: 3.2 mM Na_2CO_3, 1.0 mM $NaHCO_3$, 0.7 ml/min, 25ºC, 20 µL of sample volume, 11.3 MPa. A standard chromatogram was prepared with the following salts: KH_2PO_4, KCl, NaF, $NaNO_3$, Na_2SO_4 and KBr.

2.10. Microlagae lipid extraction

The lipid extraction was performed by extraction with chloroform and methanol as described by BLIGH & DYER (1959). The apolar fraction was dissolved in 1:1 hexane solution and water) and the lipids were recovered from the hexane fraction

2.11 Biochemical Oxigen Demand (BOD) and Chemical Oxigen Demand (COD)

The effluents or broths from the fed batch process of oleaginous yeast and after its use in cultivation of microalgae *Chlorella vulgaris* were analysed in order to evaluate BOD and COD possible reductions due to algae action. The methodologies used in its determinations were based on official standards of the Standard Methods of Analysis of Water and Wastewater, 2005.

3. RESULTS AND DISCUSSION

3.1 Fed-batch biorreactor fermentation

Oil production from *R. torulóides* LPB 0035 cultivation profiles are shown in Figures: 1, 2, 3 and 4 as well determinations described by Tables 2, 3, 4 and 5.

Table 2 - Fed batch cultivation profile of experiment 1.

Time (hours)	Glucose (g/L)	Biomass (g/L)	Lipids (%)	Lipids Production (g/L)
0	75	1	0	0
24	50.7	21.8	9.1	1.98
48	25.8	25.7	12.4	3.19
48.1	76.1	25.7	12.4	3.17
72	29.8	31.7	14.9	4.72
72.1	75.8	31.7	14.9	4.72
96	37.9	33.4	17.9	5.98
120	28.5	33.5	19.8	6.63
126	26.4	33.2	20	6.64

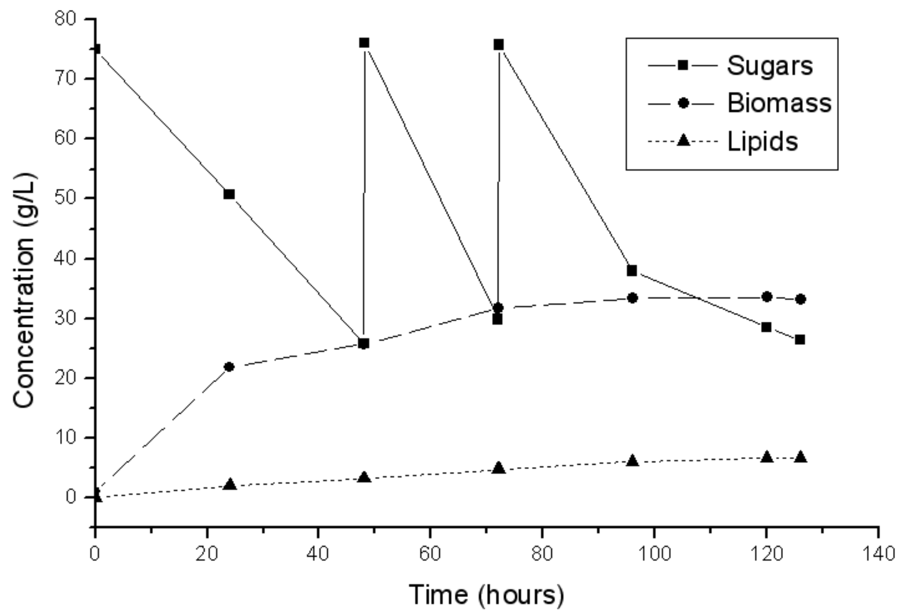

Figure 1 - Fed batch kinetic profile of experiment 1.

Table 3 - Fed batch cultivation profile of experiment 2.

Time (hours)	Glucose (g/L)	Biomass (g/L)	Lipids (%)	Lipids Production (g/L)
0	75	1	0	0
24	43.4	26.6	7.1	1.89
48	18.5	29.9	11.5	3.44
48.1	66.9	32.8	15.7	5.15
72	23.7	35.9	18.3	6.57
72.1	71.8	37.4	21.7	8.11
96	30.6	38.1	23.5	8.95
120	22.5	38.3	24.7	9.46
126	16.4	38.2	24.7	9.43

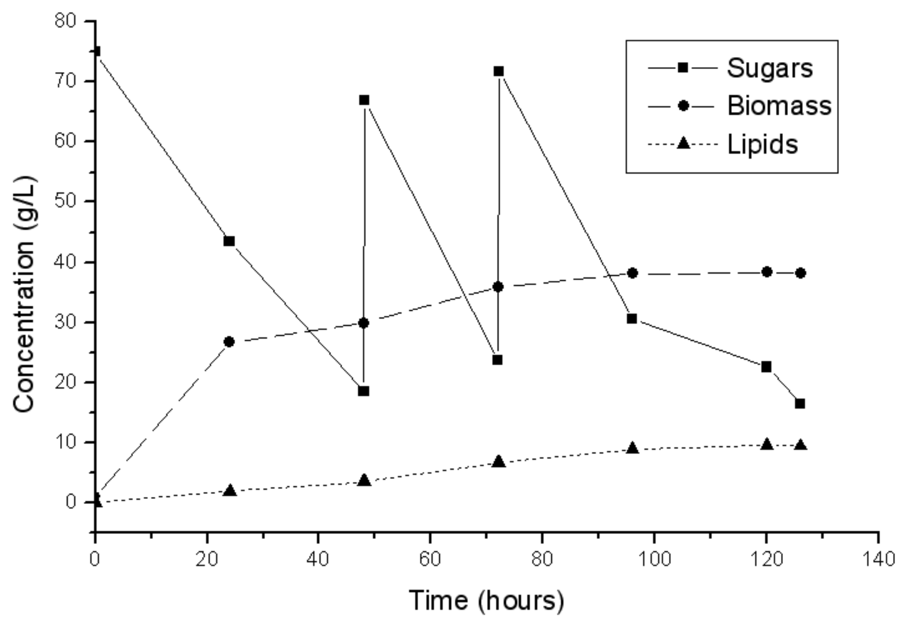

Figure 2 - Fed batch kinetic profile of experiment 2.

Table 4 - Fed batch cultivation profile of experiment 3.

Time (hours)	Glucose (g/L)	Biomass (g/L)	Lipids (%)	Lipids Production (g/L)
0	75	1	0	0
24	48	23.7	13.7	3.25
48	22.3	26.7	18.7	4.99
48.1	71.4	26.7	18.7	4.99
72	23.8	28.3	20.6	5.83
72.1	72.1	28.3	20.6	5.83
96	29.1	29.6	24.8	7.34
120	22.8	30.1	28.7	8.64
126	21.6	30.1	28.7	8.64

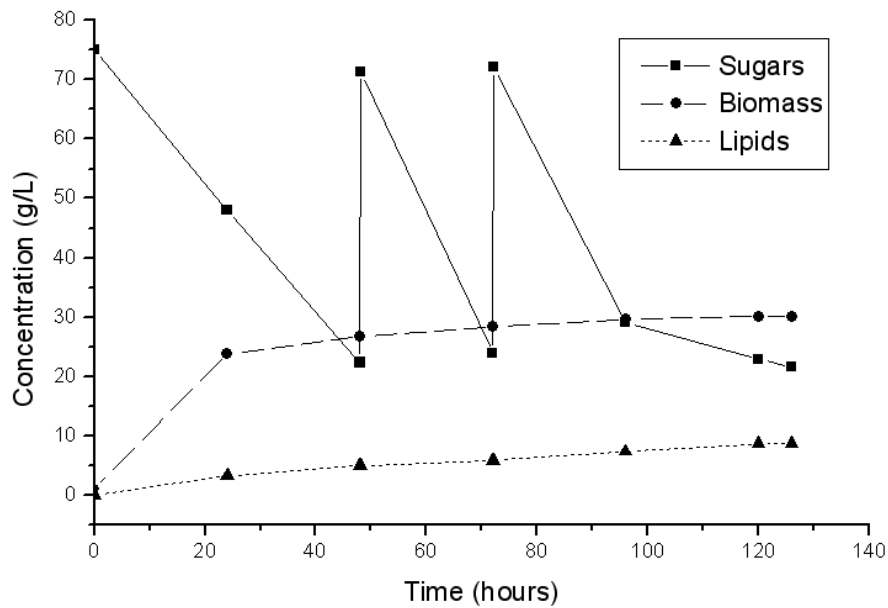

Figure 3 - Fed batch kinetic profile of experiment 3.

Table 5 - Fed batch cultivation profile of experiment 4.

Time (hours)	Glucose (g/L)	Biomass (g/L)	Lipids (%)	Lipids Production (g/L)
0	75	1.1	0	0
24	41.4	29.2	11.2	3.27
48	16.3	35.7	18.7	6.67
48.1	64.1	35.7	18.7	6.67
72	21.3	39.4	27.8	10.95
72.1	69.7	39.4	27.8	10.95
96	28.4	43.8	34.0	14.89
120	18.1	42.8	44.2	18.91
126	12.7	44.3	56,.7	25.12

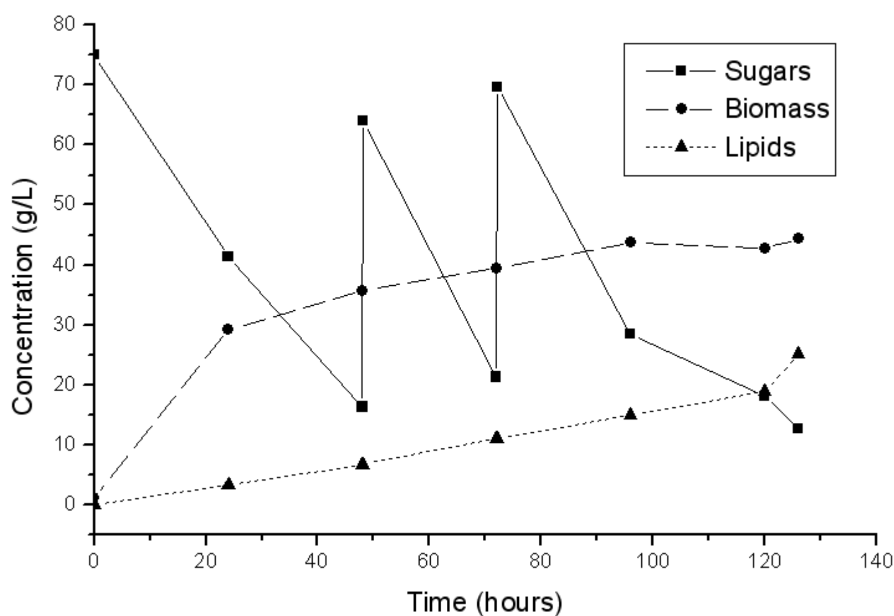

Figure 4 - Fed batch kinetic profile of experiment 4.

Li, Zhao and Bai (2007) studying High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture, observed best initial glucose concentration between 50 and 100 g/L for both lipid and biomass accumulation. Even close to 100g/L, cell growth was greatly repressed and more severe inhibitory effects were observed at even higher carbon source concentrations. However, other factors can affect fermentation performance as C/N ratio, type of nitrogen sources, presence of some elements and others.

The carbon to nitrogen (C/N) ratio is important in a biological process (LIN; LAY, 2004). Initial C/N used provided conditions to, in a first moment, estimulate cell growth. In a process performed in two stages, the C/N ratio changes during cultivation because of the nitrogen limitation applied after the first biomass production phase. YKEMA *et al.* (1988) and GRANGER *et al.* (1993) showed the importance of a high C/N ratio for lipid accumulation. The best results should be expected with C/N greater than 20 (MEESTERS; HUIJBERTS; EGGINK, 1996). In this experiment the C/N ratio was 36.32 at the beginning of all fed batch cultivations even using different nitrogen sources: urea and yeast extract. During the second phase the amount of nitrogen decreased and therefore the C/N ratio increased due to carbon feeding towards the end of the fermentation. It is important to note the use of nitrogen source even in feed process might produce a disfavored C/N ratio for lipid accumulation. In addition, this feeding operation may also be complicated.

It is noted that high dO_2 can be considered a key to the best results (LI; WEI; LIU, 2008). During experiments it was noted an increasing of rotation (up to 800 rpm) to compensate lacking of dO_2 due to high metabolic activity. Two experiments using 50% of dO_2 produced highest values of biomass and lipid accumulation. The lipids productivity (g/L/h) related to 50% dO_2 were: 0.199 (urea) and 0.075 (yeast extract). These values were lower when 30% dO_2 and lipids productivity (g/L/h) were: 0.068 (urea) and 0.052 (yeast extract).

Urea used as nitrogen source presented better results when compared to yeast extract in all experiments. During several yeast strains trials made previously (data not shown), urea also was capable to produce better results in both biomass and lipid accumulation. In fact urea is cheaper than yeast extract, has higher and expected amount of nitrogen, thus it can be a good alternative for *R. toruloides* LPB 0035 and even other yeast strains.

Li *et al.* (2010) studying single cell oil production from yeast strains in 120h fed batch process with hydrolysate of cassava starch, reported 52.9% of oil accumulation and close to 20g/L of biomass production. Zhao *et al.* (2010) researching *R. mucilaginosa* yeast strain, used hydrolysate of inulin and Jerusalem artichoke tuber extract as carbon source and reached 52,2% of oil and 19,5g/L of biomass at the end of a fed batch process. Thus, the results

obtained from this research seem to be better suitable comparing to recent publications that used yeast strains in fed batch processes.

3.2 Algae cultivation

The results presented in Table 6 and Figure 5 represent the average of three determinations for the same experimental condition in the cultivation of *Chlorella vulgaris* LPB 0033 using broth from fed batch process in photobioreactor. Figure 5 shows the evolution profile of *Chlorella vulgaris* LPB 0033 biomass from the production from oleaginous yeast.

Table 6 - Profile of *Chlorella vulgaris* LPB0033 using broth from the production of oleaginous yeast.

Time (days)	Biomass(g/L)	Lipids (%)
0	0.20 (± 0.02)	-
3	0.35(± 0.03)	2.25 (± 0.015)
7	0.59(± 0.03)	5.28 (±0.022)
12	1.22(± 0.02)	9.63 (±0.065)
17	1.52(± 0.01)	14.00 (±0.19)

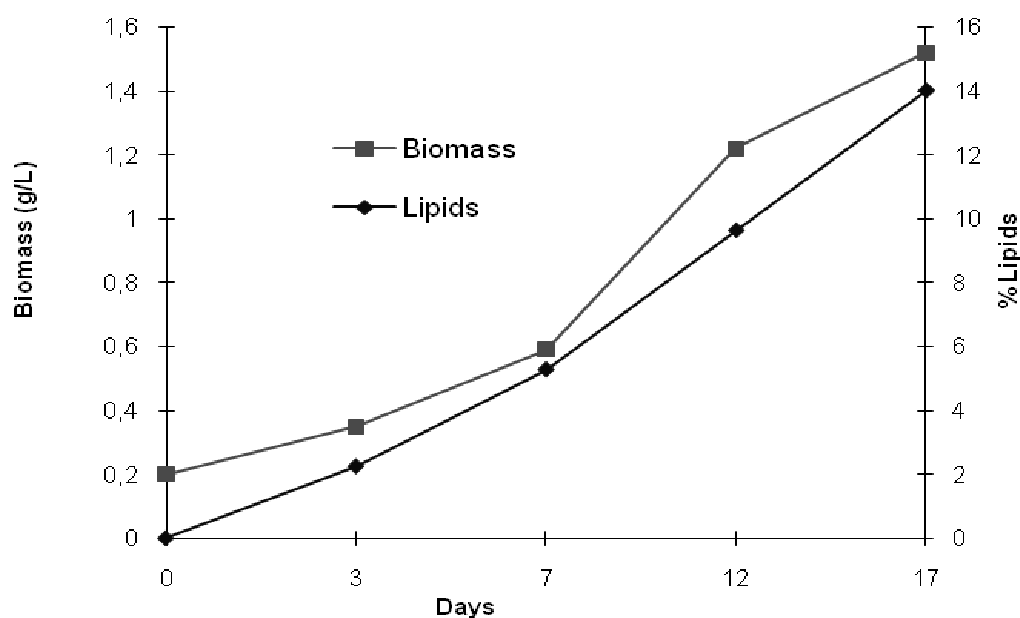

Figure 5 – C. vulgaris LPB0033 biomass and lipids formation during *C. vulgaris* LPB0033 cultivation.

Regarding lipid accumulation the experiement reached a value of 14% (± 0.19). Considering results obtained, it is possible to suggest the broth from the production from oleaginous yeast pure constitutes a potential substrate for the cultivation of the microalgae *Chlorella vulgaris* LPB 0033. YI CHIU et al (2008) found similar results regarding biomass yielding from *C. vulgaris* in a semicontinuous photobioreactor. Different yields than those presented in this experiment can be achieved, when using other samples from different cassava starch producers located in different regions, different varieties of cassava, as well as the cultivation of other species of microalgae. Anyway, *R. toruloides* LPB 0035 fed batch process is able to provide CO_2 to microalgae cultivation designing an integrated process.

3.3 Ion profile composition

Table 7 illustrates the components of the broth effluent from the oleaginous yeast production, as well as after cultivation algal composition.

Table 7 - Concentrations of anions and cations present in analyzed effluents.

Anions/Cations	Before algae cultivation	After algae cultivation
	Concentration (mg/L)	
Fluoride	0	0
Chloride	33,420.10	31378.48
Bromide	0	0
Nitrate	614.41	5.87
Phosphate	2,938.84	903.80
Sulphate	1,282.67	559.54
Amonium	1,157.92	56.27
Magnesium	300.01	158.64
Calcium	489.16	131.11
Sodium	12,393.29	8893.31

It was found that the wastewater resulting from this integrated process are excellent culture media for the cultivation of the microalgae *Chlorella vulgaris* LPB 0033 by presenting availability of nutrients that promote the production of microalgae.

3.4 Analyses of biochemical oxygen demand (BOD) and chemical oxygen demand (COD)

The results for COD and BOD for the broth from the production of oleaginous yeast and after its use in cultivation of microalgae *Chlorella vulgaris* LPB 0033 are showed in Table 8.

Table 8 – COD and BOD analyses of microalgae cultivation process.

Analysis	COD (mg/LO_2)	BOD (mg/LO_2)
Before algae cultivation	95.616.00	26.934.00
After algae cultivation	47.011.20	11.467,00

The notable reduction in parameters is probably due to the consumption of micronutrients and macronutrients present in the culture media. The cultivation of microalgae *C. vulgaris* LPB 0033 concerns the use of supernatant of algal production of microbial biomass after separating the liquid fraction of the biomass, using for example filtration or centrifugation aiming sequent oleaginous biomass production, lower values of BDO and COD are also aimed in the reuse of effluent. The objective of this experiment was to demonstrate the potential for reducing the pollution load of wastewater generated in this process through this technology.

3.5 Fatty acids analysis of Oil from R. toruloides LPB 0035 and C. vulgaris LPB 0033

After fatty acids in the extracted lipids were transmethylated and analysed by gas chromatography as described before, results in Table 9 shown that over 78% of the fatty acids from the yeast strain *R. toruloides* LPB 0035 was C16:0, C18:1 and C18:2, especially C18:1. This confirms lipids obtained in this study are very suitable feedstock for biodiesel production. Lipids from *R. toruloides* LPB 0035 also presented mainly long–chain fatty acids with 16 and 18 carbon atoms.

Table 9 - Fatty acids profile of oil obtained from *R. toruloides* LPB 0035 and *C. vulgaris* LPB0033.

Carbon	**Fatty acid**	*R. toruloides*	*C. vulgaris*
		g/100g	**g/100g**
C14:0	myristic	0.65	-
C15:0	pentadecanoic	0.21	-
C16:0	palmitic	19.12	39.32
C16:1	omega 7 palmitoleic	1.48	-
C17:0	margaric	0.51	-
C17:1	cis-10-heptadecenoic	0.15	-
C18:0	stearic	9.35	-
C18:1	omega 9 trans elaidic	0.39	-
C18:1	omega 9 oleic	50.32	28.67
C18:2	omega 6 linoleic	8.70	31.99
C18:3	omega 3 alpha linoleic	1.24	-
C20:0	arachidic	0.38	-
C20:1	omega 11 cis-11-eicosenoic	0.14	-
C20:2	omega 6 11,14 eicosadienoic	0.15	-
C22:0	behenic	0.54	-
C20:3	omega 3 cis-11,14,17-eicosatrienoic	0.07	-
C24:0	lignoceric	0.99	-
Unidentified	Unidentified	0.61	0,02

Fatty acids profile obtained in other researches from *C. curvatus* and *R. mucilaginosa* were mainly oleic (C18:1), palmitic (C16:0) and stearic (C18:0) (LI; DU; LIU,2008; ZHAO *et al.*, 2010).

3.6 Analyses of biodiesels produced from R. Toruloides LPB0035 and C. vulgaris LPB0033

Commonly, renewable triglycerides contain free fatty acids, phospholipids, sterols, water, odorants, and other lipid associates, which make the oil unsuitable for use as fuel directly in existing diesel engines (PANDEY, 2009). Physical-chemical determinations of biodiesels obtained in this research are presented in Table 10 and 11.

Table 10 – Phisical-chemicals determinations for biodiesels obtained from *R. toruloides*LPB 0033.

Determination	Method	Methil esters	Ethil esters
		Result	**Result**
Specific gravity at 20°C	ASTM D1298	880 kg/m^3	888 kg/m^3
Kinematic viscosity at 40°C	ASTM D445	4,90 mm^2/s	5,51 mm^2/s
Water	ASTM 2709	380 mg/kg	410mg/kg
Contamination	EN 12662	3 mg/kg	3 mg/kg
Flash point	ASTM D93	170 °C	170 °C
Carbon residue 100%	ASTMD189	0,01 %	0,01 %
Total sulphur	EN 14596	0 mg/kg	0 mg/kg
Phosphorus	ASTM 4951	0 mg/kg	0 mg/kg
Ester content	EN 14103	>97 % mass	>97 % mass
Free glycerin	EN 14105	0,01 % mass	0,02 % mass
Total glycerin	EN 14105	0,130 % mass	0,165 % mass
Monoglycerides	EN 14105	0,45 % mass	0,40 % de massa
Diglycerides	EN 14105	0,17 % mass	0,17 % mass
Triglycerides	EN 14105	0,09 % mass	0,09 % mass
Methanol or ethanol	EN 14110	0,06 % mass	0,07 % mass
Oxidation stability at 110°C	EN 14112	>6 hours	>6 hours
Sulfated ashes	ASTM D874	0,01 % mass	0,01 % mass
Acid index	ASTM D664	0,1 mg KOH/g	0,14 mgKOH/g
Cold filter plugging point	ASTM D2500	7 a 8	8 a 9
Iodine index	EN 14111	68	65
Calorific value	ASTM E711	9160 kcal/kg	9150 kcal/kg

Table 11 – Phisical-chemicals determinations for biodiesels obtained from *C. vulgaris* LPB 0033.

Determination	Method	Methil esters	Ethil esters
		Result	Result
Specific gravity at 20°C	ASTM D1298	879 kg/m^3	879 kg/m^3
Kinematic viscosity at 40°C	ASTM D445	4,99 mm^2/s	4,99 mm^2/s
Water	ASTM 2709	350 mg/kg	350 mg/kg
Contamination	EN 12662	3 mg/kg	3 mg/kg
Flash point	ASTM D93	165 °C	165 °C
Carbon residue 100%	ASTMD189	0,01 %	0,01 %
Total sulphur	EN 14596	0 mg/kg	0 mg/kg
Phosphorus	ASTM 4951	0 mg/kg	0 mg/kg
Ester content	EN 14103	>97 % mass	>97 % mass
Free glycerin	EN 14105	0,01 % mass	0,01 % mass
Total glycerin	EN 14105	0,140 % mass	0,140 % mass
Monoglycerides	EN 14105	0,48 % mass	0,48 % mass
Diglycerides	EN 14105	0,17 % mass	0,17 % mass
Triglycerides	EN 14105	0,09 % mass	0,09 % mass
Methanol or ethanol	EN 14110	0,06 % mass	0,06 % mass
Oxidation stability at 110°C	EN 14112	>6 hours	>6 hours
Sulfated ashes	ASTM D874	0,01 % mass	0,01 %mass
Acid index	ASTM D664	0,1 mg KOH/g	0,18 mgKOH/g
Cold filter plugging point	ASTM D2500	7 - 8	7 a 8
Iodine index	EN 14111	68	70
Calorific value	ASTM E711	9160 kcal/kg	9180 kcal/kg

Biodiesels produced was submitted to ASTM and EN Official methods. These parameters comply with the limits established by ASTM related to biodiesel quality (ANTOLIN *et al.*, 2002). The physical and fuel properties of biodiesel from yeast oil in general were comparable to those of standard diesel fuel. The results suggested this fed batch cultivation and further processes made in order to obtain final product, could be a feasible and effective method for the production of high quality biodiesel from *R. toruloides* LPB 0035 and *C. vulgaris* LPB 0033. The biodiesel from present studied yeast oil could be a competitive alternative to conventional diesel

4. CONCLUSION

The results demonstrate that this study, which combines bioengineering and transesterification is potentially feasible and efficient for the production of high quality and low-cost biodiesel from microbial oil. Over 87.6% of the fatty acids from the yeast strain *R. toruloides* LPB 0035 cultivated in the hydrolysate of cassava tuber was C16:0, C18:1 and C18:2, especially C18:1 (50,32%).The same behaviour was observed regarding microalgae *C. vulgaris* LPB 0033 where fatty acids C16:0, C18:1 and C18:2 reached 99,98% of total composition. Therefore, the oils from a good oil feedstock for biodiesel production as physical chemical results demonstrated. However, reduction of material pretreatment costs and uses of other city and agricultural residues and matters must be investigated even to provide other carbon sources from lignocelullosic biomasses.

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CONCLUSION

The biodiesel from oleaginous microbial biomass can be obtained within hours in an integrated technological process, controlled and predictable. Moreover, the diesel oil is a non-renewable fuel in the short term, since the oil takes millions of years to form. As raw materials for obtaining oleaginous microbial biomass, stand out among various carbon sources simple and complex. Glucose can be obtained from the hydrolysis of natural polymers such as starch and / or cellulose. In particular, may be potential sources of raw cassava and other tuber crops. One of the positive aspects of using these crops for biodiesel would be the possibility of microbial intercropping cassava and sweet potatoes for example without a loss in production of both crops, a fact that could increase the productivity of starch per hectare. In competition with this source of sugars for the biotechnological application processes include sugarcane and its derivatives featuring a monoculture. This scheme brings cultivate environmental disadvantages because exhausts the soil over time and reduces biodiversity. Social disadvantages occur because it reduces the use of manpower in the countryside and rural populations. Furthermore, the monoculture of sugarcane can lead to some economic disadvantages, because it presents enormous risks, as a single disease or pest or the fall of the price of the product on the market could compromise the entire region production. The restoration of degraded ecosystems is receiving increasing importance in facing current increasing environmental crisis and decreasing quality of life of human populations and overall nature. The sustainable cultivation of cassava has potential application of positive results in degraded areas. Because a tuber crop is able to adapt very well to impoverished areas, the use of these varieties to restore degraded areas should be considered. Finally, we described a simple fed-batch process for lipid production by oleaginous yeast *R. toruoides* strain with high cell density. The process features a nutrient-rich initial medium, sole carbon source feeding is convenient for large-scale operation. Furthermore, effluents generated by fed-batch process (CO_2 and residual liquid) can be used to microalgae growth improving good quality oil/biodiesel production and reducing pollutants. This treated effluent can be re-used as broth for fed batch producing and even for new microalgae sequential cultivations. This strategy

significantly improves biomass and lipid productivity and will be useful for further engineering of feasible microbial lipid production processes using an integrated process.

SUGGESTIONS TO FUTURE RESEARCHES:

- Test other carbon sources like sweet potato or other amylaceous raw materials;

- Hydrolyse fibrous residual content of raw material in order to improve the reducing sugars generation from chemical or enzymatic conversions;

- Studying the engineering of microorganisms capable to accumulate lipids to get overall higher process yielding;

- Carrying out other microalgae cultivation systems;

- Evaluate the influente of different ratios of air/CO_2 on microalgae yielding;

- Studying the sequential algae cultivation in order to obtain a final wastewater of even better quality;

- Verify the feasibility of using residual matter of hydrolysed cells to feed or human consumption due to its high protein content.

APPENDIX

PATENT
INPI 221008039408
(22/12/2010)

“Processo Integrado para produção de lipideos/oleos de origem microbiana e algal a partir de tubérculos amiláceos.”

Main inventor:
Carlos Ricardo Soccol. Ph.D.
Co-inventors
Luiz Gustavo Lacerda, MSc.
Dolivar Coraucci Neto. MSc.

< Uso exclusivo do INPI >

Espaço reservado ao protocolo

Espaço para etiqueta

DEPÓSITO DE PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas

1. Depositante (72):

1.1 Nome: **UNIVERSIDADE FEDERAL DO PARANÁ**

1.2 CNPJ/CPF: **75095679000149**
1.3 Endereço completo: **RUA XV DE NOVEMBRO, 695 - CENTRO CURITIBA – PR**
1.4 CEP: **80020-310**
1.5 Telefone: **(41) 3310-2699** 1.6 FAX: **(41) 3310-2760**
1.7 E-mail: **inovacao@ufpr.br**

(X)continua em folha anexa

2.Natureza: Patente de invenção (x)Invenção ( )Modelo de Utilidade ( )Certificado de Adição

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: Patente de Invenção

3.Título da Invenção ou Modelo de Utilidade ou Certificado de Adição(54):
PROCESSO INTEGRADO PARA A PRODUÇÃO DE LIPÍDIOS/ÓLEOS DE ORIGEM MICROBIANA E ALGAL A PARTIR DE TUBÉRCULOS AMILÁCEOS

( )continua em folha anexa

4.Pedido de Divisão: do pedido Nº Data de Depósito:

5.Prioridade: ( ) interna ( ) unionista
O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor:
( ) **Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)**

6.1 Nome: Carlos Ricardo Soccol
6.2 Qualificação: Ph.D 6.3 CPF: 27558479991
6.4 Endereço completo: Rua Pedro Demeterco, Jardim das Américas, Curitiba - PR
6.5 CEP: 81530-320
6.6 Telefone:(41) 3361-3191 6.7 FAX:
6.8 E-mail: soccol@ufpr.br

(x)continua em folha anexa

Formulário 1.01 – Depósito de Pedido de Patente ou de Certificado de Adição (folha 1/2)

7.Declaração na forma do item 3.2 do Ato Normativo nº 127/97

( ) 7.1 Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

8. Declaração de divulgação anterior não prejudicial (Período de Graça):
(art. 12 da LPI e item 2 do AN nº 127/97)

9. Procurador (74):

9.1 Nome:
9.2 CPF/CNPJ: 9.3 API/OAB:
9.4 Endereço Completo:
9.5 CEP:
9.6 Telefone: ( ) 9.7 FAX: ( )

10. Listagem de sequências biológicas (documentos anexados) (se houver):
Listagem de sequências em arquivo eletrônico: nº de CDs ou DVDs (original e cópia).
Código de controle alfanumérico no formato de código de barras: fl.
Listagem de sequências em formato impresso: fls.
Declaração de acordo com o artigo da Resolução INPI nº 228/09: fls.

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

X	11.1 Guia de Recolhimento	fls.	X	11.5 Relatório descritivo	fls.
	11.2 Procuração	fls.	X	11.6 Reivindicações	fls.
	11.3 Documentos de Prioridade	fls.	X	11.7 Desenhos	fls.
	11.4 Doc. de contrato de trabalho	fls.	X	11.8 Resumo	fls.
X	11.9 Outros que não aqueles definidos no campo 11 (especificar) DOU				fls.

12. Total de folhas anexadas (referentes aos campos 10 e 11): fls.

13. Declaro, sob penas da Lei, que todas as informações acima prestadas são completas e verdadeiras.

Prof. Dr. Zaki Akel Sobrinho
Reitor

Curitiba, 22/12/2010
Local e Data

Assinatura e Carimbo

Formulário 1.01 – Depósito de Pedido de Patente ou de Certificado de Adição (folha 2/2)

ANEXO DE CONTINUAÇÃO – FORMULÁRIO 1.01

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7. Inventor (72):

 Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s) (art. 6º § 4º da LPI e item 1.1 do Ato Normativo nº 127/97)

Nome: Luiz Gustavo Lacerda
Qualificação: Doutorando
Endereço: Rua Luiz Alberti, 190
CEP: 81220-050
Telefone: (41) 8432-0018
E-mail: luizgustavo@up.com.br

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7. Inventor (72):

 Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s) (art. 6º § 4º da LPI e item 1.1 do Ato Normativo nº 127/97)

Nome:Dolivar Coraucci Neto
Qualificação: Engenheiro Químico
Endereço: Av. Independência, 3320 – Sala 24, Alto da Boa Vista – Ribeirão Preto, SP
E-mail: dolivar.couracci@ourofino.com

ANEXO DE CONTINUAÇÃO – FORMULÁRIO 1.01

7. Depositante (72):

1.1 Nome: Ourofino Participações e Empreendimentos S.A.
1.2 CNPJ/CPF: 07.065.512/0001-85
1.3 Endereço completo: Rodovia Anhanguera SP 330, km 298 – Cravinhos/SP
1.4 CEP: 14.140-000
1.5 Telefone: (16) 3518.2031
1.6 FAX: (16) 3518.2000

Jardel Massari
Presidente
Grupo Ouro Fino