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Biotechnology: use of available carbon sources on the planet to generate alternatives energy

Biotecnologia: uso de fontes de carbono disponíveis no planeta para geração de energia alternativa

Biotecnología: uso de las fuentes de carbono disponibles en el planeta para generar energías alternativas

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Abstract
Biotechnology has been an essential tool in the search for solutions and in the optimization of bioprocesses associated with issues of human, plant, animal, energy and also the balance of ecosystems on planet Earth. The objective of this research was to present an unconventional substrate (cellulose), in abundance on the planet, to be used as a substitute source of carbon and energy for biotechnology processes, with the possibility of increasing industrial production of biomass and energy. As basis for the research, an extensive literature review and quantitative and qualitative analyzes were carried out. Genetic Engineering techniques were used to enable the yeast *Saccharomyces cerevisiae* for partial cellulose degradation, through the use of genetic transformation methods to insert a plasmid carrying the cellobiohydrolase cDNA. It was found that the recombinant and biologically active cellobiohydrolase protein was expressed and excreted in haploid and diploid laboratory yeast strains. The analyzes allowed the visualization of cellulolysis halos around colonies of recombinant strains grown in solid YPD medium with 1% microgranular cellulose. The recombinant clones derived from the haploid lineage yielded in average of 1.70 mg ART/mL, while recombinant clones derived from the diploid lineage produced in average of 2.05 mg ART/mL.
Keywords: Bioethanol; Cellobiohydrolase; Cellulose; Genetic engineering; Yeast.

Resumo
A biotecnologia tem sido uma ferramenta fundamental na busca de soluções e na otimização de bioprocessos relacionados às questões de saúde humana, vegetal, animal, energia e também ao próprio equilíbrio de ecossistemas do nosso planeta. O propósito desta pesquisa foi justamente usar um substrato não convencional em abundância no

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planeta, a celulose, para ser usado como fonte de carbono e energia alternativos aos processos biotecnológicos, com a possibilidade de ser incrementado na produção industrial de biomassa e energia. Como base da pesquisa, realizou-se uma revisão bibliográfica extensa e análises do tipo quanti e qualitativas. Foram utilizadas técnias da Engenharia Genética para capacitar a levedura *Saccharomyces cerevisiae* à degradação parcial da celulose, utilizando o método de transformação genética por eletroporação para inserir um plasmídeo carreador do cDNA da celobiohidrolase. Verificou-se que a proteína celobiohidrolase recombinante e biologicamente ativa foi expressada e excretada em linhagens da levedura *Saccharomyces cerevisiae* de laboratório haplóide e diplóide que receberam o plasmídeo com a introdução do inserto cDNA - celobiohidrolase. As análises permitiram a visualização de halos de celulolise ao redor de colônias das linhagens recombinantes crescidas em meio sólido YPD com 1% de celulose microgranular. Os clones recombinantes derivados da linhagem haplóide produziram na média1,7 mg de ART/mL; enquanto que os clones recombinantes derivados da linhagem diplóide produziram na média 2,05 mg de ART/mL.
Palavras-chave: Bioetanol; Celobiohidrolase; Celulose; Engenharia genética; Levedura.

Resumen
La biotecnología ha sido una herramienta fundamental en la búsqueda de soluciones y en la optimización de bioprocesos relacionados con cuestiones humanas, vegetales, animales y energéticas, así como con el propio equilibrio de los ecosistemas de nuestro planeta. El propósito de esta investigación fue precisamente utilizar un sustrato no convencional y abundante en el planeta, la celulosa, para ser utilizada como fuente alternativa de carbono y energía a los procesos biotecnológicos, con posibilidad de incrementarse en la producción industrial de biomasa y energía. Como base para la investigación, se realizó una extensa revisión bibliográfica y análisis cuantitativos y cualitativos. Se utilizaron técnicas de ingeniería genética para permitir que la levadura Saccharomyces cerevisiae degradara parcialmente la celulosa, utilizando el método de transformación genética por electroporación para insertar un plásmido portador del cDNA de celobiohidrolasa. Se verificó que la proteína celobiohidrolasa recombinante y biológicamente activa se expresó y excretó en cepas de levadura de laboratorio haploides y diploides Saccharomyces cerevisiae que recibieron el plásmido con la introducción del inserto de CDNA - celobiohidrolasa. Los análisis permitieron visualizar halos de celulólisis alrededor de colonias de las cepas recombinantes cultivadas en medio sólido YPD con 1% de celulosa microgranular. Los clones recombinantes derivados del linaje haploide produjeron un promedio de 1,7 mg ART/mL; mientras que los clones recombinantes derivados del linaje diploide produjeron un promedio de 2.05 mg de ART/mL.
Palabras clave: Bioetanol; Celobiohidrolasa; Celulosa; Ingeniería genética; Levadura.

1. Introduction

The growing world-wide population, currently 7.7 billion people (ONU, 2018), must be accompanied by the innovations and technologies applied by science in a rhythm able of meeting the needs of humanity that lives primarily by the exploitation of resources of planet Earth. Several researches have been carried out in the most varied university centers in the country (Brazil) and in the world in search of answers capable of meeting demands in a sustainable way. Among these, they detach the necessities and food and energetic deficiencies that become basic to the life processes and for the economic and social development of the nations.

However, as a consequence in the search for solutions, possibilities arise for the generation of new products and the establishment of new markets through biotechnology, which consists in the use of cellular systems for the development of processes and products of economic and social interest. Among cellular systems, fungi are of great biotechnological interest, probably constituting the living beings that most generate products and processes of fundamental importance for the well-being of the population (Azevedo, 2002). In this context, means have been sought capable of replacing or improving the existing raw material sources for the production of products, food, as well as in the generation of energy. With regard to the latter, Brazil has been one of the pioneer countries that has been successful in obtaining clean energy, from the decade 1970 with the global oil crisis that led to the development of the National Proalcohol Program (Kohlhepp, 2010). Thus, Brazil is considered one of the world leaders in the production of clean energy through the use of yeast biomass in fermentation processes of sucrose from sugar cane (*Saccharum officinarum* L.) for the production of ethanol (bioethanol/biofuel).

According to market data released by the National Supply Company (Conab, 2019) on sugar and ethanol production, Brazil produced around 18 billion liters of fuel ethanol in the 2008/09 harvest and 33.14 billion liters of ethanol in the 2018/19

harvest that represents 70 billion real in the national economy. The production of Brazilian ethanol (bioethanol), from the sugar cane raw material, has been considered a sustainable process since ethanol is produced from a renewable (vegetable) source and has been widely recognized in the world as a viable and economically important alternative in comparison to fossils fuels used (Brazil, Ministry of Mines and Energy/Energy Research Company, 2007).

In Brazil, due to the use of fermenting microorganisms with the ability to transform substrates into ethanol (Buckeridge, 2008), the sugar-energy agro-industrial sector (sugar-alcohol) stands out with strong expansion and economic development in several Brazilian states (Barros et al., 2019). According to Limayem and Ricke, 2012; one of the alternatives for increasing the production of industrial products would be through the use of plant biomass, which is a rich source of carbon and energy, highlighting the sources of forest resources, agro-industrial residues, herbaceous materials, municipal and industrial waste.

Ethanol production from sugar cane can be done in two ways: as a first generation (1G) and second generation (2G) product (Florencio, et al, 2017). According to Monteiro, 2016, 1G ethanol is that produced through sucrose (composed of a glucose molecule and a fructose molecule). This has a low production cost and has been widely used today. On the other hand, 2G Ethanol is produced from sugar cane biomass and the process has been developed on a smaller scale due to the high cost of production compared to the first, due to the need to carry out the hydrolysis processes of the lignocellulosic substrates before alcoholic fermentation (Furlan, 2015; Robak & Balcerek, 2018).

In this way, the constant search for new yeast strains with high fermentative potential becomes evident, whether through bioprospecting (Basso et al., 2008), the advancement of genetic engineering (Paulo et al., 2019), by metabolic engineering (Ostergaard et al., 2000) or by evolutionary engineering (Basso et al., 2011). To promote the second generation processes of ethanol, it is necessary to pre-treat vegetable biomass, sugarcane bagasse, with enzymes capable of hydrolyzing cellulose (N-glucose molecules in β-1.4 bond) in order to supply glucose for the aerobic and fermentative metabolism of yeasts *S. cerevisiae* (Santos et al., 2012).

In this context, the cellulose molecule is composed of cellobiose disaccharide units, which is composed of N glucose molecules in β-1.4 glycosidic bond followed by the elimination of water through hydroxyls linked to carbon 1 and 4. Thus, the cellulose is an important substrate of vegetable origin with high energy potential, as its basic molecular structure consists of a linear homopolymer of 8000-12,000 glucose monomers joined by β-1.4 type glycosidic bond (Enari, 1983; Zhang et al., 2006; Voet and Voet, 2010; Costa Junior, et al, 2013; Nelson and Cox, 2018) having high stability and requiring the action of enzymes to promote their degradation. Therefore, cellulose is a natural substrate of high industrial potential because it consists of N molecules of glucose which is the primary source of carbon and energy for the metabolic processes of most living organisms, like the human being himself. Thus, due to the source of carbon and energy in cellulose, it, after being hydrolyzed, may serve as a substitute substrate for conventional sources of raw material to be used in industrial bioprocesses for the production of biomass and energy.

Much of the research that can revolutionize the processes of generating products, food and energy in a way that is beneficial to the planet, is being directed to groups of microorganisms that have long gone unnoticed by humanity, although already used for millennia of years. Among these, living in the most diverse aquatic and terrestrial environments from the tropics to the Arctic and Antarctic regions are those belonging to the phylum of the Fungi which besides having a great physiological variety and dimensions, from visible to the naked eye to visible only through the microscope, can make possible impacts positive to sustainable development when used appropriately in the generation of useful products, such as: in the synthesis of insecticidal substances that help in pest control, in the bioremediation of environments degraded by chemical pollutants and by the degradation of cellulolytic compounds and of the lignin itself present in plants (Maia, 2010; Silva & Coelho, 2006).

Among the single-celled fungi currently used in industries with high efficiency for the production of biomass, alcoholic beverages and energy (single cell protein and ethanol) are yeasts which are single-celled fungi, presenting a well-defined cell membrane, not very thick in young and rigid cells in adult cells, of variable constitution with predominance of carbohydrates and less protein and lipids and with optional metabolism (aerobic and anaerobic). Thus, in the absence of oxygen, yeasts promote fermentation, hydrolyzing the glucose molecule to produce energy (ATP), decarboxylating and reducing pyruvate to release CO_2 and form ethanol (Da Silva, 2016).

Differences in the assimilation of carbon sources and in the ability to ferment different sugars are important criteria in the taxonomy for the identification of yeasts (Walt and Yarrow, 1984). According to Albergaria and Arneborg (2016), several yeast genera and species are biochemically capable of converting sugars to ethanol and carbon dioxide, as well as generating other important metabolites, such as: glycerol, acetate, succinate, pyruvate, higher alcohols, esters of great industrial importance and energetic. However, these yeasts do not naturally contain the gene for the synthesis of cellulases, as is the case with the worldwide known yeast *Saccharomyces cerevisiae*.

According to Landry, et al., 2006, for an ethanol production from lignocellulosic biomass, it would be necessary for yeasts to be capable of hydrolyzing cellulose and adjusting to the adverse conditions of the industrial fermentation medium, such as: low pH, medium osmotic pressure due at high rates of fermentable carbohydrates at high levels of ethanol. In function of these abiotic factors and other production factors, the yeast *Saccharomyces cerevisiae* has been chosen for the production of second-generation ethanol. However, although this yeast is capable of efficiently fermenting glucose, the production of second-generation ethanol is limited by the lack of cellobiose metabolism by *S. cerevisiae* cells that do not naturally have the genes to synthesize cellulase enzymes and therefore, are unable to hydrolyze this disaccharide (Lee et al., 2013).

Another characteristic that highlights the yeast species of the genus *Saccharomyces* for bioprocesses concerns the ability to not develop pathogenicity to healthy human beings - "Generally Regarded As Safe" (GRAS), in addition to presenting high metabolic versatility and easy genetic manipulation. For these reasons, yeasts are being tested and used in laboratory bioprocesses, including genetic engineering, for the production of bioactive substances, including enzymes. Among the most described enzymes are: proteases, cellulases, lipases, cellobiases, xylanases, esterases, glycosidases (Brizzio et al., 2007; Landell & Valente, 2009).

Aiming at the use of plant biomass, the main enzymes capable of degrading the cellulose molecule are cellulases that constitute an enzymatic complex capable of acting on lignocellulosic materials and promoting their hydrolysis until the release of glucose monomers. Cellulases are classified according his acting place in the substrate cellulosic and are divided into three major groups: endonucleases that cleave internal bonds of the cellulosic fiber; exoglucanases that act on the outer region of cellulose, and β-glycosidases that hydrolyze glucose-soluble oligosaccharides (Teery, 1997; Lynd et al, 2002; Castro & Pereira Jr., 2010).

With the use of the glucanases described in the previous paragraph, the process of hydrolysis of cellulose (plant biomass) would occur as follows: the endoglucanases hydrolyze amorphous regions of the cellulose and catalyze the hydrolysis of internal sites producing celloligosaccharides. These celloligosaccharides are subsequently hydrolyzed by the action of cellobiohydrolases enzymes (exoglucanases) generating cellobiose dimers. Finally, cellobiose is converted to glucose by the action of the enzyme β-glycosidase (Phitsuwan et al., 2013).

The exoglucanases are divided into: a) glucanhydrolase, which is a little reported enzyme and has a highly important cellulosic fiber hydrolysis strategy, as it is capable of releasing glucose directly from the polymer; b) cellobiohydrolase that participates in the primary hydrolysis of the fiber, performing the phenomenon of physical disruption of the substrate, leaving the crystalline regions exposed to cellulases and releasing the disaccharide cellobiose.

The activity of cellobiohydrolases consists of penetrating the crystalline regions of the microfibrils to degrade the cellulose polymer from the ends generated by the endoglucanases. Synergism also occurs between different subtypes of cellobiohydrolases, since there are enzymes that attack the reducing ends (CBHI) and others that attack the non-reducing ends (CBHII) of cellulose fibers (Lynd et al., 2002).

Therefore, cellobiohydrolases act in progressive way on the reducing (CBHI) and non-reducing (CBHII) ends of cellulose, they act with greater affinity for insoluble or microcrystalline cellulose, producing a slow and gradual reduction in the degree of polymerization by the release of glucose and, mainly, cellobiose as a final product. According to Martins, 2017, cellobiohydrolases have the active site in the form of a tunnel through which the cellulose chain penetrates and undergoes catalysis of its terminal glycosidic bonds. It also highlights that these enzymes generally suffer inhibition due to the high concentration of their own final hydrolysis product, cellobiose.

Cellobiose is composed of two units of glucose joined by β-1.4. The degradation of cellobiose by celobiase (β-glucosidase) results in the formation of glucose, this end product being of hydrolysed of cellulose the fermentable sugar with industrial applications (Fuentefria and Valente, 2004). According to Chi et al., 2009, cellulase enzymes (endoglucanases, exoglucanases and cellobiases) that are currently being used in cellulose degradation processes are produced mainly by filamentous fungi.

The group of exoglucanases has been little mentioned, however, these enzymes have the ability to hydrolyze the cellulose biopolymer from his extremities. Cellobiohydrolases (CBH) are among the most efficient enzymes as cellulitic agents and have the systematic name 1.4-β-D-glycanocelobiohydrolase. Such enzymes play an essential role in the decomposition processes of cellulolytic compounds, and can be divided into two types: type I cellobiohydrolase, CBH I, which has the function of hydrolyzing reducing terminals, while type II, CBH II, hydrolyzes terminals non-reducing. The hydrolysis product of these enzymes is cellobiose, (Den Haan et al., 2007; Bommarius et al., 2008; Den Haan et al., 2013; Sorensen et al., 2013).

An interesting feature of CBH (cellobiohydrolase) is its ability to promote the phenomenon known as amorphogenesis that involves the physical disruption of the cellulolytic substrate by increasing the exposure of the interstitial regions, facilitating the primary catalysis of the non-reducing terminals of the cellulosic fiber and oligosaccharides to release the cellobioses, in addition to presenting the ability to attack the reducing terminals. During the CBH activity there is an increase in the cellulose hydrolysis rates, leaving the polymer more exposed to other cellulases (Castro and Pereira Jr., 2010).

According to Castro and Pereira Jr., 2010, during the enzymatic hydrolysis processes of lignocellulosic materials, high hydrolysis rates are observed in the initial stages of the reaction and, during the process, there is a reduction in these rates, as well as a reduction in activity of cellobiohydrolases, therefore, the substrate matrix sites are already saturated by biocatalysts. Wang et al., 2010, highlighted that the most accessible regions of the polysaccharide are hydrolyzed in the initial stages of hydrolysis, while the most resistant fractions remain intact throughout the process.

Among the main sources of cellulases already used commercially, we highlight those from the filamentous fungi *Trichoderma reesei*, *Aspergillus niger* and *Humicola insolens* (Sandgren et al., 2005; Khademi et al., 2002). Haki & Rakshit, 2003, highlighted the degradation of crystalline cellulose, insoluble in water, by thermostable cellulases. Therefore, in the production of cellulolytic enzymes, the fungi *Chaetomium thermophile*, *Thermoascus aurantiacus*, *Sporotrichum thermophile* and *Humicola grisea* var. *thermoidea* (Maheshwari et al., 2000).

Considering its important cellulolytic activity, the fungus *Trichoderma harzianum*, has a great potential for application to hydrate biomass. However, the cellulases of this filamentous fungus were still not characterized in depth. Cellobiohydrolase I (CBHI) is the main cellulolytic enzyme applied by *Trichoderma* sp., currently being one of the most investigated cellulases for biotechnological applications in the area of biofuels. A CBHI hydrolyzes crystalline cellulose releasing as soluble units of cellobiose, that is, it becomes a key enzyme for the production of fermentable sugars from

biomass. In the fungus *Trichoderma* sp., a proportion of cellobiohydrolase (CBHI) is very expressive, reaching approximately 80% of the cellulases produced by this microorganism (Saloheimo and Pakula, 2012; Lo and Ju, 2009).

The cellulose biodegradation system as a source of carbon and energy for cellular metabolism (Teeri, 1997; Watanabe and Tokuda, 2010; Andlar et al, 2018), by influence synergistically in the action of cellulases to cellulosic substrate to solubilize it in glucose monomers; cellobiohydrolases act on the reducing and non-reducing terminals of cellulose fibers with the release of cellobioses. The endoglucanases hydrolyze the cellulose chains randomly and are responsible for the rapid solubilization of the cellulosic polymer, due to the cellulose fragmentation into oligosaccharides. While β-glycosidases hydrolyze cellobiose and release glucose from the non-reducing ends (Kleman-Leyer et al., 1996).

In nature, the main microorganisms that synthesize cellulolytic enzymes are not economically viable to bioprocesses, including:*Trichoderma reesei, Trichoderma viride, Humicola grisea, Phanerochaete chrysosporim, Macrophomina phaseolina, Butyrivibrio fibrisolvens, Cellulomonas fimi, Pseudomonas fluorescens subsp. cellulosa; Clostridium thermocellum e Ruminococcus flavefaciens* (Araújo et al., 1983; Azevedo et al., 1990; Cheng et al., 1990; Cullen and Kersten, 1992; Li and Renganathan, 1998; Rabinovich et al., 2002). In this context, for cellulose to be used as a source of carbon and energy in bioprocesses, it would be necessary to promote a great reconciliation between: the microorganisms that synthesize cellulases and that are not viable for bioprocesses, the current methods of industrial production and the microorganisms traditionally used by industries in bioprocesses (do not synthesize cellulases).

Among the natural cellulolytic microorganisms, the fungi of the *Humicola* genus have a wide geographical distribution on the planet with the soil as their traditional habitat. They are classified as Deuteromyces of the family Dermatiaceae (White and Downing, 1953). These fungi are characterized by secreting thermostable cellulolytic enzymes, presenting an optimum activity temperature around 50ºC. Still, they are able to tolerate changes in the pH range of the substrates, according to Chaves and Brune, 1989; Monti et al., 1991; Gaur et al., 1993. According to Peralta et al., 1990; Takashima et al., 1996; *Humicola grisea* secretes β-glycosidase in high concentrations, making it an efficient natural cellulose degrading fungus.

Several genes coding for cellulases of the fungus *Humicola* have already been expressed in other organisms that have a high capacity to produce and secrete these heterologous proteins at high levels, such as *Aspergillus* (Christensen et al, 1988; Dunn-Coleman et al, 1991). Other researchers also reported the enzymatic activity of these fungi on different sources of cellulosic substrates (Takashima et al., 1996; Schülein, 1998).

Among the works developed in the field of genetic engineering of yeasts capable of promoting the use of cellulose as a substrate (source of carbon and energy), the following stand out: Van Rensburg et al., 1998, genetically constructed strains of *Saccharomyces cerevisiae* that expressed and secreted a complex of cellulotitic genes, which gave the strains of genetically modified yeast high enzymatic power in the hydrolysis of cellulosic materials (carboxymethylcellulose, hydroxyethylcellulose, barley glucans, birch bark xylans, polypectates, cellobiosis and methyl-β-D-glycopyranosides). The genes used were: BGL 1 (β-1.4-Glycosidase) from *Endomyces fibuliger*; END1 (β-1.4-Endoglucanase) from *Butyrivibrio fibrisolvens*; CBH1 (β-1.4-Glucanocelobiohydrolase) from *Phanerochaete chrysosporium*; CEL 1 (Celodextrinase) of *Ruminococcus flavefaciens*. Okada et al., 1998, constructed recombinant strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* that showed endoglucanase activity of 17 and 400mU/mL of CMC medium (carboxymethyl cellulose), respectively, through the insertion of the EGIII gene from *Trichoderma reesei* QM9414, a low molecular weight endoglucanase.

Another very important aspect in microorganisms is the term "Single Cell Protein" (SCP) which is used to designate inactivated cells of microorganisms that are used as a source of biomass and can be used in food, both human and animal; among these microorganisms, yeasts, bacteria, fungi and algae stood out. This "single cell protein" terminology was first used

by Professor Carol Wilson of the Massachusetts Institute of Technology (M.I.T) in the 1950s to improve the image of protein of microbial origin (Ware, 1977).

In this context, the development of technologies for biomass production, according to Brown, 1968, arose from the need to produce protein food in large quantities, in reduced areas and, in a shorter time, to meet the needs of poor populations. This concern received greater importance in the period after World War II (1948), when there was a significant change in food production in the world, shaking economic and trade relations mainly between developed and underdeveloped countries such as those in Asia and South America that had their economies based on primary production, agriculture and livestock, which assured them negotiating power in the international market. In addition, the consequences of the war and population growth projections for the beginning of the new millennium were added, estimated at around 8 billion people, currently at 7.7 billion individuals and with a growing projection to 9.7 billion by 2050, according to a new United Nations report (2019).

In the 1980s (20th century), according to World Health Organization data, 12 million people died each year from hunger, malnutrition and starvation-related diseases. Half of these deaths directly affected children under the age of 5 years (Miller, 2005). According to the Food and Agriculture Organization of the United Nations - FAO (2000 and 2002) and the State Report on World Food Security and Nutrition, the number of hungry people in the world is around 821 million, the study reveals that out of every nine people, one has fallen victim to hunger.

After so many years, hunger is still a current problem that is arousing more and more interests within the scientific community, industries and governments for the need to find another food source capable of meeting human needs so as not to depend solely on agriculture, fishing and cattle raising. In this sense, the proposal to use microbial biomass in the human and animal diet is well founded on the advantages of rapid production and the nutritional value of this food, as can be seen in Tables 1 and 2.

Table 1 - Time for doubling of body mass.

Organisms	**Mass doubling time**
Bacteria and yeasts	10 - 120 minutes
Fungi and algae	2 - 6 hours
Grasses and other plants	1 - Two weeks
Chickens	2 - 4 weeks
Pigs	4 - 6 weeks
Cattle	1 - 2 months
People	0,2 - 0,5 years

Source: Israelidis, 1982 (Food Technology Institute, Greece); Moore, *et al* (2020).

Table 2 - Average nutritional composition of microorganisms in percentages (%).

Microrganisms	**Yeasts**	**Algae**	**Fungi**	**Bacterias**
Protein	45 - 55	40 - 70*	30 - 45	50 - 65
Lipideos	2 - 6	7 - 20	2 - 8	1,5 - 3,0
Nucleic acids	6 - 12	3 – 8	7 - 10	8 - 12
Ashes (minerals)	5 - 9,5	8 - 10	9 - 14	3 - 7

**Spirulina platensis*. Source: Miller *and* Litsky, 1976; Usharani, *et al* (2012).

Among the microorganisms that have been traditionally used in the production of human food for millennia and without generating toxicity, the yeast of the Saccharomyces genus stands out. The biomass derived from yeast of the species

Saccharomyces cerevisiae presents a composition of amino acids comparable to the best usual protein sources (Han, *et al*., 1971; Gamba, *et al*, 2020), such composition is described in Table 3.

Table 3 - Comparative data on amino acid composition between the yeast *S. cerevisiae*, wheat, eggs and cow's milk and Fermented cow's milk (cow's milk kefir) in grams of amino acid/100g of protein.

Amino acid	*S. cerevisiae*	Wheat	Egg	Cow milk	Fermented cow's milk
Lysine	7.7	2.8	6.3	7.8	0.67
Threonine	4.8	2.9	5.0	4.6	1.30
Methionine	1.7	1.5	3.2	2.4	0.09
Cysteine	-	2.5	2.4	-	-
Triptophan	1.0	1.1	1.6	-	-
Isoleucine	4.6	3.3	6.8	6.4	0.88
Leucine	7.0	6.7	9.0	9.9	1.04
Valine	7.1	4.4	7.4	6.9	1.01
Phenylalanine	4.1	4.5	6.3	4.9	0,52
Histidine	2.7	-	-	-	1.07
Arginine	2.4	-	-	-	0.10

Source: Han, *et al*, (1971); Gamba, *et al*, (2020).

Furthermore, it is noticed that the yeast biomass is rich as a vitamin nutrient, presenting in its cell composition excellent levels of the B vitamins, such as: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, folic acid, biotin and P-acid-aminobenzoic (Vasey and Powell, 1984).

The biomass of yeasts of the *Saccharomyces* genus is already being used in animal feed, according to the personal communication of the chemical engineer of the Sugar and Alcohol Plant JARDEST S.A/São Paulo-Brazil in which the yeast dough discarded in the fermentation process of ethanol production goes through a drying and packaging process to be exported to Japan in order to serve in fish food (Grael, 1998). A great advantage in "SCP" production when compared to traditional agricultural food production is verified by the independence of variable production factors, such as: climate, land availability, soil fertility, cost with inputs, pesticides and veterinary medicines. It should also be noted that the production of cellular biomass does not affect the environment; therefore, its production is sustainable and ecologically correct.

There are currently 70,000 species of fungi already described, which represents only 5% of the estimated number of fungal species (Azevedo and Pizzirani-Kleiner, 2002) and only 1% of bacterial species are known (De Melo et al, 2002). Thus, the Brazilian microbial biodiversity is an excellent research material of high biotechnological potential, fruit of our immense and ancient natural laboratory called Biosphere (Planet Earth).

Genetic engineering techniques, more specifically recombinant DNA technology (DNAr), began to be defined in the early 1970s with the use of cloning vectors; in general, plasmids and viral genomes used restriction enzymes that allowed DNA to be cut at well-defined points in order to isolate fragments of specific nucleic acids (genes) that could be introduced into the genome of another organism (compatible host cell). Each restriction enzyme has a specific cutting pattern of the DNA molecule, resulting in a series of genomic fragments that can be isolated, cloned and sequenced. Plasmids are generally found in bacteria and in some yeasts and have the ability to replicate themselves autonomously, generally possessing genes that confer resistance to antibiotics such as ampicillin and tetracycline. It is these genes that distinguish recombinant host cells, which possess DNA, from those that do not. Also, when the recombinant cell divides, it also has the faculty of duplicating the molecule of the inserted DNA fragment.

For the production of recombinant enzymes on an industrial scale, a wide range of organisms or host cells can be used. The choice is usually made based on criteria, such as: the cost-benefit ratio, the solubility of the protein, the ease of purifying the protein, the activity of the protein and whether, at the end, the protein will be crystallized (Hartley, 2006).

Basically, genetic manipulation comprises four phases: choosing the DNA fragment to be used, cutting this fragment (restriction enzyme), transferring and inserting it into the genome of a given cell, and finally, selecting the recombinant cells that contain the molecules of DNA from the desired clone (Candeias, 1991).

The discovery of new active metabolites of microbial origin is also another major challenge that could bring substantial benefits to human needs and the biosphere. In this way, fungi are considered essential for the maintenance and balance of many terrestrial ecosystems, that is, they are important for their sustainability and biodiversity. In this context, it is necessary to emphasize the importance of Biotechnology for obtaining various substances through the manipulation of fungi in obtaining new products for the benefit of human health and environmental balance (Abreu et al., 2015).

Fungi are efficient producers and secretors of enzymes and are therefore able to degrade a wide spectrum of natural and also synthetic substrates. This metabolic adaptability makes fungi widely used in biotechnological processes as bioreactors or as a source of enzymes, proteins or peptides of industrial interest (Hurst et al., 2002).

From a molecular point of view, industrial strains of microorganisms - when compared to laboratory strains - are genetically more complex and have no desired stability in their haploid state, and it is common to find aneuploidies in industrial yeasts. On the other hand, these strains have evolved to a better adaptation in different environments or ecological niches, modified or not, due to anthropic actions. This adaptation process is called "domestication" and may have been responsible for certain genetic characteristics of industrial strains over time, such as the varying number of ploidy and chromosomal polymorphisms (Lucena et al., 2007).

Modification of the genetic makeup of industrial strains for better adaptation to the industrial environment usually occurs through events such as: Mitotic recombination between homologous sequences, mitotic crossing-over and gene conversions. These changes tend to be fixed in a lineage as a result of selective pressure for the genetic characteristics that best meet those environmental conditions (Leite, 2008). Lynd et al, (2002) described the presence of extra chromosomes in industrial strains and with it over-expression of genes, an adaptation of this type of strain that could give another advantage to laboratory strains (Zaldivar et al, 2002).

Among the fungi considered to be single-celled, the yeast *S. cerevisiae* has been described as the ideal microorganism for genetic manipulation (Recombinant DNA Technology-TDR), aiming at the production of heterologous proteins, due to its safe handling and the ability to perform modifications post-translational: acetylation, phosphorylation, glycosylation that are often necessary to guarantee the biological activity of the protein (Romanos, et al., 1992; Hinnen et al., 1995). This quality has been facilitated after the publication of the complete yeast genome (Ostergaard et. al., 2000).

In the field of DNAr Technology, Azevedo et al., 1990, performed the characterization of a cellobiohydrolase gene (cbh1.1) from *Humicola grisea* var. *thermoidea* that presented 60% homology with a *T. reesei* cellulase gene. Subsequently, the cellobiohydrolase 1.4 gene (cbh1.4) was identified (Poças-Fonseca et al., 1997) and it was found that the protein encoded by this gene was more abundant in the supernatant fungus culture when grown in sugarcane bagasse (De Paula et al., 2003). The cbh1.4 gene was cloned and expressed in *Saccharomyces cerevisiae* (Benoliel et al, 2010) and in *Pichia pastoris* (Godbole et al, 1999) for the purpose of enzyme characterization. The regulation of the expression of these cellobiohydrolase genes was analyzed, growing *Humicola grisea* var. *thermoidea* in different carbon sources and it was proven that cbh1.4 presented a constitutive expression, while cbh1.1 presented a distinct regulatory mechanism (Poças-Fonseca et al., 2000). It was observed that in the recombination process the insertion of an integrative plasmid into the yeast chromosome would occur in the following ways:

a) Integration by addition: when there is a single recombination or "crossing-over" event and the plasmid integrates into the chromosome being flanked by direct repetitions of the homologous region and when a large amount of integrative

genetic material is used in the transformation, multiple tandem insertions may also occur, probably due to the repetitions of the recombination events (Romanos et al., 1992).

b) Replacement integration: when recombination events occur leading to a replacement of a chromosomal sequence by its corresponding homologous sequence present in the plasmid, without the need for integration of the entire plasmid. This phenomenon was interpreted as being the result of a double crossing-over or gene conversion (Hou et al., 2009).

2. Material and Methods

The work consisted of an applied research of an experimental nature with qualitative and quantitative approaches, in addition to a broad bibliographic review to base the applications of innovative and sustainable biotechnological products from molecular constructions by Genetic Engineering techniques, which aim to genetically improve microorganisms used in bioprocesses. Laboratory analyzes - preparation of microorganisms, extraction, isolation and purification of DNA, restriction analysis, genetic construction of the recombinant plasmid, cloning, electrophoresis and verification of the activity of the heterologous cellobiohydrolase protein in yeast colonies - followed the methodologies described by Sambrook et al., 1989; Boyer & Roulland-Dussoix, 1969; Sikorski and Hieter, 1989; Miller, 1959.

2.1 Microorganisms / Strains Used

a) Escherichia coli XL1-Blue: *rec*A1, *end*A1, *gyr* A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac*, [F' *prob*AB, *lac* ZΔM15, *Tn*10(tet^r)]. (Sambrook *et al.*, 1989).

b) E. coli DH5α: *sup*E44; Δ *lac*U 169 (80 *lac*ZDM 15); *hsd*R17; *rec* A1; *end*A1; gyrA96 ; *thi*-1; *rel*A1 (Sambrook *et al.*, 1989).

c) E. coli HB 101: *sup*E44, *hsd*S20, *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, *xyl*-5, *mtl*-1 (Boyer & Roulland-Dussoix, 1969).

d)Saccharomyces cerevisiae YPH 252 haploide**:** MATα; *ura*3$^-$52; *lys2*$^-$801^{amber};*ade*2$^-$101^{ochre}; *trp*1-Δ1; *his*3-Δ200; *leu*2- Δ1. (Sikorski and Hieter, 1989).

e) Saccharomyces cerevisiae YPH 274 diploide a /α; *ura*3$^-$52/ *ura*3$^-$52, *lys*2$^-$801^{amber}/ *lys*2$^-$801^{amber}; *ade*2$^-$101^{ochre}/*ade*2$^-$101^{ochre}; *trp*1$^-$Δ1/*trp*1$^-$Δ1; *his*3$^-$Δ200/*his*3$^-$Δ200; *leu*2$^-$Δ1/ *leu2*$^-$Δ1 (Sikorski and Hieter, 1989).

2.2 Plasmids Used

YEp352:Ap^R URA3 (Hill *et al*., 1986); **PHIL-D2**:Ap^R, containing the *CBH* 1.4 cDNA encoding the cellulase 1.4-beta-cellobiohydrolase protein ("EC: 3.2.1.91"), (Azevedo, *et al*, 1990); YEpAEL2: 7.1kb, Ori de Col E1, 2μm, Amp^R, URA3, cloning sites for the restriction enzymes *Eco* RI, *Bam* HI, *Pst* I, *Hind* III, *Bgl* II, Promoter and Terminator of transcription of the enzyme alcohol dehydrogenase I of *Saccharomyces cerevisiae* (*ADH* I) .; **YEp352-PADHI:CBH** (8.54Kbp/figure 1).

2.3 Culture Media

a) Yeasts: YPD, SD, SD + amino acids; b) Bacteria: LB, SOB, SOC; (Difco, Biobrás).

2.4 Solutions and buffers

Ampicillin, biotin, leucine, nitrogenous bases, glycerol, glucose 20%, K_2HPO_4 1M, sorbitol, TE, solutions (I, II, III), PEG 8000, NaCl, RNAse A, TEB 10x, ethidium bromide, chlorofane, chlorofil, congo red 1%.

2.5 DNA treatment

Specific enzymes - New England Biolabs, Gibco-BRL Products.

2.6 Transformation of bacteria and yeasts

Cell porator Pulser (Bio-Rad Laboratories, Richmond, CA-USA).

2.7 DNA analysis and Electrophoresis

Extraction, isolation, purification, restriction, electrophoresis (Sambrook et.al., 1989).The visualization of the gel was carried out in a UV light transilluminator, in the Eagle Eye digital photo documentation (Stratagene®).

2.8 Analysis of recombinant *S. cerevisiae* clones

a) Qualitative: According to Teather and Wood, 1982; the recombinant yeasts were identified by a medium zone (cellulolysis halos) around the colonies. The recombinants yeasts have grown in selective media and tested positive for congo red/iodine were chosen for determination of cellulolytic activity. The recombinant yeasts were inoculated in in solid medium Agar-microcrystalline cellulose (Avicel PH101): 1% microcrystalline cellulose; 0.2% $NaNO_2$; 0.1% K_2HPO_4; 0.05% $MgSO_4$; 0.05% Peptone; 2% agar; 1% congo red indicator. The yeasts were inoculated by the sowing technique in solid medium and incubated in a biological oven at 28ºC/48hours, later, the plates were revealed by the addition of 1% potassium iodide solution and, after 5 minutes, washed with water to verify the halo formation.

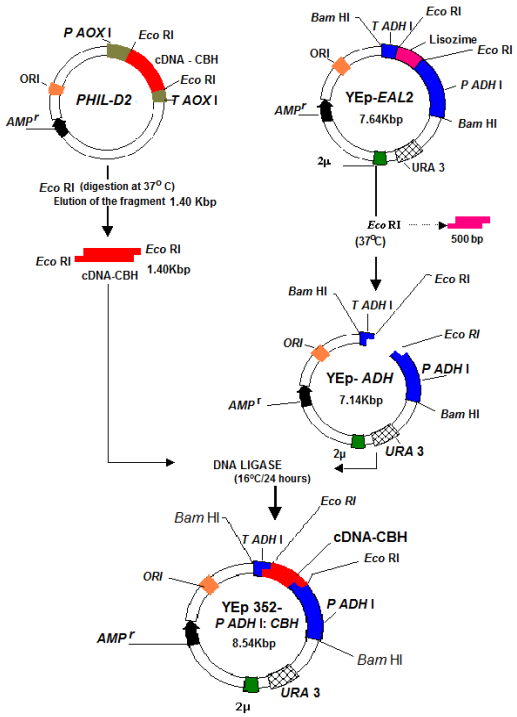
b) Quantitative: According to Miller, 1959; cellulolytic activity recombinant yeasts was determined using the 3.5-dinitrosalicylic acid (DNS).

3. Results

3.1 Construction of the Recombinant Plasmid YEp352-PADHI:CBH

The 1.4kb EcoRI-DNA fragment containing CBH 1.4 cDNA (cellobiohydrolase) from *Humicola grisea* var. *thermoidea* was extracted from the PHIL-D2 vector (Azevedo *et al*., 1990) and inserted into the EcoRI restriction site of the episomal plasmid YEp352:PADHI, derived from YEpEAL2 (Grael, 1998), being flanked by promoter and gene transcription sequences of alcohol dehydrogenase I (ADH I) of *S. cerevisiae*. The molecular construction steps of the YEp352-PADHI:CBH 8.54kb vector, performed in vitro, are shown in Figure 1.

Figure 1 - Schematic representation of the strategy used for molecular construction of plasmid YEp352-PADH I:CBH (8.54kbp).

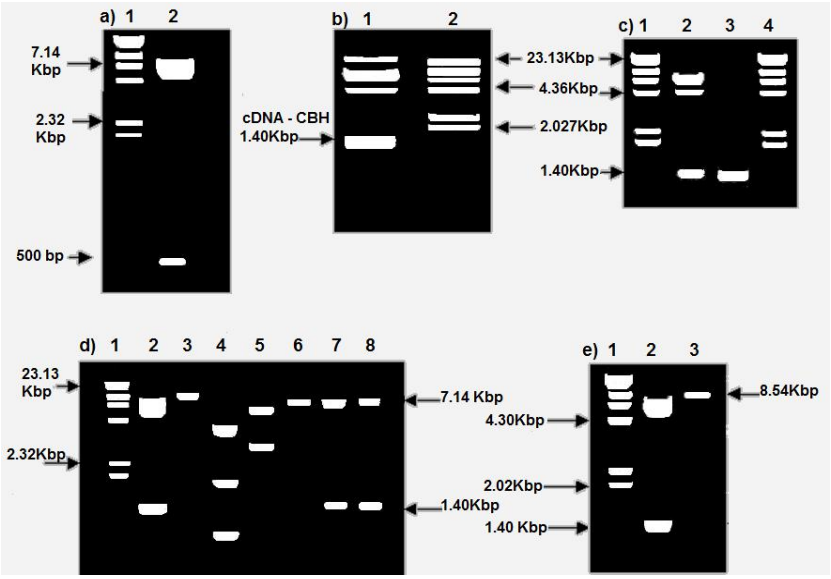


Source: Authors.

3.2 Restriction analysis of recombinant clones with the Plasmid YEp352-PADH I:CBH (8.54kpb):

The ligation mixture was used for the genetic transformation of *E. coli* DH5α cells. 19 colonies of transforming Ap^R bacteria were obtained. Restriction analyzes of recombinant plasmids, extracted from these clones, are shown in figure 2 (a-e).

Figure 2 - Photographs of 0.8% agarose gels, in 0.5x TBE buffer, after electrophoresis run. **a**) 1: molecular weight marker λ-*Hind* III; 2: digestion of plasmid YEpAEL2 with *Eco*RI, releasing the 500bp fragment (lysozyme); **b**) 1: digestion of PHIL-D2 with *Eco*RI, releasing the 1.4 kbp DNA fragment, corresponding to the CBH cDNA; 2: *λ*-Hind III; **c**) 1: *λ*-Hind III; 2: digestion of plasmid DNA with *Eco*RI, releasing the DNA fragment corresponding to the CBH cDNA (1.4 kbp); 3: 1.4 kbp eluted and purified DNA fragment, corresponding to the CBH cDNA; 4: molecular weight marker *λ*-Hind III; **d**) 1: *λ*– Hind III; 2 to 8: plasmid DNA of the bacterial transformants obtained with the YEp352-PADHI binding mixture and CBH cDNA; **e**) 1: *λ* -Hind III; 2: digestion of plasmid DNA isolated from the bacterial clone, presenting the cloning vector YEp352-PADHI:CBH digested with *Eco*RI; 3: vector YEp352-PADHI:CBH linearized with *Pst*I (8.54kpb).



Source: Authors.

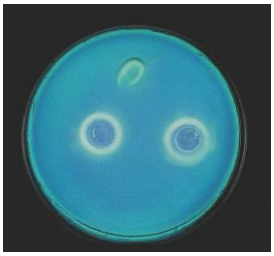
3.3 Quantitative evaluation of recombinant *Saccharomyces cerevisiae* clones excreting the cellobiohydrolase enzyme

The clones, randomly chosen from each of the recombinant haploid (YPH252) and diploid (YPH274) strains, excreted the biologically active cellobiohydrolase. Analysis of ART (total reducing sugar) obtained by the hydrolytic action of cellobiohydrolase in the supernatant growth culture on filter paper yielded the following results: a) recombinant clones derived from haploid lineage produced 1.62; 1.65 and 1.82 mg of ART/mL; b) recombinant clones derived from diploid lineage produced 2.04; 2.05 and 2.07 mg of ART/mL. The control strains (haploid and diploid) did not show any recombinant production of reducing sugar.

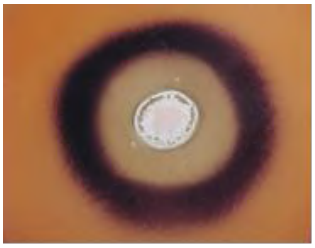
3.4 Qualitative analysis of *Saccharomyces cerevisiae* recombinant clones

The excretion and activity of the recombinant protein on the cellulolytic substrate has been proven by viewing halos. Thus, the recombinant yeast strains that received plasmid YEp352-PADHI:CBH presented transparent halos around the colony (indicative of enzymatic activity), while the untransformed strain used as a negative control did not present cellulolytic halo, figures 3(a) and (b).

Figure 3 - Images of Petri dishes containing recombinant yeast colonies. **a)** Petri dish with YPD + 1% microcrystalline cellulose (Avicel-PH101), stained with Congo red 1%, (blue contrast).



a)



b)

Source: Authors.

Recombinant lines YPH252 and YPH274 present halos of cellulolytic activity (left and right of the central region of the plate), the negative control line does not present the cellulolytic halo (on top of the plate). **b)** Image of Petri dish (close-up) with YPD + 1% microcrystalline cellulose (Avicel-PH101), shown with recombinant yeast strain *S. cerevisiae* and stained with 1% congo red indicator, showing halo of cellulolytic activity.

4. Discussion

Knowing that the yeast of the *Saccharomyces cerevisiae* species naturally does not have genes for the synthesis of cellulases, but that it has been the yeast in preference to fermentative bioprocesses both for the production of bioethanol, energy cogeneration and even for the increase of human and animal food (single cell protein), it can be said that this scientific work added another positive point, among the various efforts of world researchers, in the area of genetic engineering to enable the enzymatic hydrolysis of cellulosic raw material through the heterologous expression of cellulases by *Saccharomyces cerevisiae*. In this sense, the initial results obtained and presented in this work could prove the success of cloning, through: a) the constructed episomal plasmid; b) in the transformation of yeast strains; c) in the expression of the cellobiohydrolase 1.4 cDNA of *Humicola grisea* var. *thermoidea*; d) the biological activity of recombinant cellobiohydrolase. The expression and excretion of the cloned CBH cDNA gene product in URA 3 episomal plasmid was analyzed under the regulation of the *S. cerevisiae* ADHI promoter and transcription terminator. In addition, the yeast mating pheromone signal sequence, factor MFα-

1, was used to ensure the excretion of recombinant cellobiohydrolase originating from *Humicola grisea* var. *thermoidea*, verifying the enzymatic action of this cloning product on the cellulosic material through the direct visualization of cellulolysis halos around the recombinant yeast colonies, whereas the wild-type strain was not, as indicated by the absence of a transparent circle in solid medium; similarly other works: Liu *et al*, 2018, who were successful in cloning recombinant *Saccharomyces* yeasts containing the expression cassettes of β-glucosidase (BG) enzyme, the cellobiohydrolase (CBH) and the endoglucanase (EG).

Specifically, regarding the construction of the episomal vector of transformation YEpE352-PADHI: CBH 1.4, it is possible to affirm: I) It was successful in transforming the haploid and diploid yeast strains with the vector YEpE352-PADHI: CBH 1.4; II) It was found that cellobiohydrolase was excreted by the recombinant clones of *S. cerevisiae*; III) Cellobiohydrolase was biologically active (visualization of the cellulolysis halos around the recombinant yeast colonies); IV) The action of cellobiohydrolase present in the supernatants of recombinant cultures provided the cellulose hydrolysis generating total reducing sugar (ART) as the final product.

In this sense, through the use of biotechnology and genetically improved microorganisms that are considered harmless to human health, it is possible to infer that cellulose could be used in bioprocesses to generate biomass - production of single cell protein (SCP) in short time, including alternative foods for humans and animals, since yeasts have nutritional properties in amino acids similar to traditional foods such as cow's milk, wheat, eggs. Would also be useful for bioprocesses in the transformation of fermentable carbohydrates for the production of alcoholic beverages and the generation of clean energy (environmentally correct), such as the production of bioethanol (ethyl alcohol) and energy cogeneration.

5. Final Considerations

The objectives of this work were successfully achieved, from the stages of elaboration and construction of the episomal cloning vector (YEpE352-PADHI: CBH 1.4), as well as in the yeast transformation process until the expression of the heterologous protein with cellulolytic enzymatic activity proven by qualitative and quantitative analyzes performed.

In a future perspective, it is intended to carry out tests to optimize the production of enzymes by recombinant yeast strains and to analyze the enzymatic activity. In addition, complementary genetic information for the expression and excretion of the enzyme glucosidase (BGL) may be added to this recombinant yeast strain, carrier of cDNA of cellobiohydrolase. Thus, this double recombinant will be able to perform the total hydrolysis of cellulose in glucose molecules. In this way, recombinant *Saccharomyces cerevisiae* yeast will be built, which can be used in countless biotechnological processes from the most abundant and available substrate on planet Earth as a source of carbon and energy: cellulose.

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