AMZY TANIA VALLENAS ARÉVALO

BIOLOGICAL DEGRADATION OF CYANIDE USING NATIVE BACTERIA ISOLATED FROM A CASSAVA-PROCESSING EFFLUENT

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Biological degradation of cyanide using native bacteria isolated from a cassava-processing effluent

Corrected Version

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This work is dedicated to Manuel B. Arévalo (MD),
my greatest inspiration in heart, brain, and soul.
I'll miss you forever, grandpa.

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“Kept in a small bowl, the goldfish will remain small. With more space, the fish can grow double, triple or quadruple its size.”

John August based on Daniel Wallace’s “Big Fish”

RESUMO

O cianeto (CN) pode estar presente no ambiente devido a atividades humanas, incluindo mineração de ouro, galvanoplastia e produção de nylon. Durante o processamento industrial da mandioca, o cianeto é liberado nos efluentes devido à presença de glicosídeos cianogênicos na polpa. O cianeto é tóxico para a maioria dos organismos vivos, pois inibe a ação da citocromo c oxidase e interrompe a cadeia de transporte de elétrons durante a respiração celular. Por esse motivo, os efluentes contendo cianeto precisam ser tratados antes da descarga ao ambiente, o que pode ser feito por meio de métodos químicos, físicos ou biológicos. Nesse contexto, este trabalho avalia a capacidade de degradação de cianeto de bactérias nativas isoladas de um efluente de processamento de mandioca e as melhores condições para a remoção biológica de cianeto. No total, dezesseis bactérias resistentes à presença de cianeto foram isoladas, identificadas por a técnica de espectrometria de massas MALDI-TOF, e testadas individualmente em a sua capacidade de degradação de cianeto. Oito espécies diferentes foram identificadas entre as cepas isoladas. Entre elas, quatro cepas pertencentes às espécies *Klebsiella oxytoca*, *Bacillus pumilus*, *Corynebacterium glutamicum* e *Serratia marcescens* foram selecionadas para avaliar sua suscetibilidade ao pH e à concentração inicial de cianeto. Os resultados mostraram que a degradação do cianeto foi inibida quando o pH foi acima de 9 e que maiores concentrações iniciais de cianeto resultaram em menores porcentagens de degradação de cianeto. No entanto, as cepas ainda mantem a sua capacidade de degradação mesmo em altas concentrações iniciais de cianeto de 1000mg.L^{-1}. A cepas isoladas de *Klebsiella oxytoca* e *Serratia marcescens* foram selecionadas para otimização do processo de degradação, analisando a influência da fonte de carbono, temperatura e velocidade de rotação. Ambas cepas apresentaram maiores taxas de degradação ao usar acetato de sódio como fonte de carbono e incubadas a 30°C. A velocidade de agitação não mostrou efeito na degradação do cianeto por *Klebsiella oxytoca*, mas favoreceu a degradação pela cepa *Serratia marcescens*. Sob condições ideais de incubação, as cepas isoladas de *Klebsiella oxytoca* e *Serratia marcescens* podem degradar o cianeto de uma solução sintética de 150 mg.L^{-1} de cianeto livre, pH 8, usando acetato de sódio como fonte de carbono em 43 e 41% após 24horas de incubação, respectivamente, mostrando seu potencial para ser usado na biodegradação de efluentes que contêm cianeto.

Palavras-chave: Cianeto. Biodegradação. Bactéria. *Klebsiella oxytoca*, *Serratia marcescens*.

ABSTRACT

Cyanide (CN) can be present in the environment due to human activities including gold mining, electroplating, and nylon production. During the industrial processing of cassava, cyanide is liberated in effluents due to the presence of cyanogenic glycosides in the pulp. Cyanide is toxic for most living organisms since it inhibits the action of the cytochrome c oxidase and stops the electron transport chain during cellular respiration. Because of this, cyanide-containing effluents need to be treated prior to discharge to the environment, which can be achieved by chemical, physical or biological methods. In this context, this work evaluates the cyanide-degradation capacity of native bacteria isolated from a cassava-processing effluent and the best conditions for the biological removal of cyanide. In total, sixteen cyanide-resistant bacteria were isolated, identified by mass spectrometry technique MALDI-TOF, and tested individually for their ability to degrade cyanide. Eight different species were identified among the isolated strains. Four of them, belonging to the *Klebsiella oxytoca*, *Bacillus pumilus*, *Corynebacterium glutamicum,* and *Serratia marcescens* species, were selected for further assays to assess their susceptibility to pH and cyanide initial concentration. Results showed that cyanide degradation was inhibited when pH was above 9 and that higher initial cyanide concentrations resulted in lower cyanide degradation percentage. However, strains could still keep their degradation capacity even in high initial cyanide concentrations of 1000mg.L^{-1}. Isolated *Klebsiella oxytoca* and *Serratia marcescens* were thus selected for optimization of the degradation process by analyzing the influence of carbon source, temperature and rotation speed. Both strains showed greater degradation rates when using sodium acetate as a carbon source and incubated at 30°C. Agitation speed did not show an effect on cyanide degradation by *Klebsiella oxytoca*, but enhanced degradation by *Serratia marcescens* strain. Under optimal incubation conditions, isolated *Klebsiella oxytoca* and *Serratia marcescens* strains could degrade cyanide from a synthetic 150mg.L^{-1} free-cyanide solution, pH 8, using sodium acetate as a carbon source by 43 and 41% after 24hour of incubation, respectively, showing their potential to be used in the biodegradation of cyanide-containing effluents.

Keywords: Cyanide. Biodegradation. Bacteria. *Klebsiella oxytoca*, *Serratia marcescens*.

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1 INTRODUCTION

Cyanide is a widely used chemical that can be present in the environment by natural sources such as cassava, apple seeds, and almonds; but can also be released into the environment by mining, plastic production, cassava-processing, or electroplating industries (POULTON, 1990; MUDDER; BOTZ, 2004).

Cyanide is toxic for most living organisms since it inhibits the action of the cytochrome c oxidase and stops the electron transport chain causing hypoxia at cellular level (ATSDR, 2006; HAMEL, 2011). For chemical analyses purposes, cyanide can be measured as free-cyanide (HCN and CN^-); Weakly Acid Dissociable (WAD Cyanide), which includes free-cyanide and cyanide complexes with Ni, Cd, Cu and Zn; and Total Cyanide, which includes WAD cyanide and strong cyanide complexes with Au and Fe (BOTZ, 1999). The toxicity of these chemical species is based on their potential to release free-cyanide since this is the most toxic form of cyanide (JOHNSON, 2015). Because of this, careless discharge to the environment of cyanide-containing wastes and wastewaters represent an environmental and health risk, and therefore, they must be treated to comply with legislation.

During cassava flour production, cyanide is liberated from the cyanogen glycosides present in cassava (MCMAHON; WHITE; SAY, 1995). The most representative of these glycosides is linamarin, which is decomposed by the linamariase enzyme, and transformed into acetone and free-cyanide in a two-steps reaction (BEHERA; RAY, 2017). Hence, cassava-processing wastewater is the most significant waste in quantity during cassava industrial processing and it can content toxic cyanide concentrations that represent a challenge for their treatment and reuse (BALAGOPALAN, 2002).

Cyanide-containing wastes and wastewaters can be treated using natural degradation, chemical or biological processes (DASH; GAUR; BALOMAJUMDER, 2009). Currently, chemical processes such as alkaline chlorination, oxidation with hydrogen peroxide, and INCO SO_2/Air process are the most widely used. However, these processes can generate other pollutants, use expensive reagents, require complex facilities or do not get to treat some

cyanide compounds (BOTZ, 1999; DASH; GAUR; BALOMAJUMDER, 2009; KUYUCAK; AKCIL, 2013).

In this context, microbial degradation of cyanide has appeared as a cheap, simple and viable alternative technology for cyanide treatment (DASH; GAUR; BALOMAJUMDER, 2009). During the biological treatment of cyanide, it is consumed within the metabolic pathways of microorganisms transforming it into ammonia, formate, formide, and other non-toxic products (GUPTA; BALOMAJUMDER; AGARWAL, 2010). Metabolic pathways for cyanide treating include hydrolytic, reductive, oxidative and synthesis/substitution reactions catalyzed by enzymes (EBBS, 2004).

Microorganisms can attenuate the concentration of cyanide in effluents by using it as a whole source of nitrogen or carbon, or by decomposing cyanide in presence of other nutrients (GUPTA; BALOMAJUMDER; AGARWAL, 2010). Cyanide-degrading species include bacteria, fungi and algae organisms that are able to grow in cyanide-containing media, and have been previously isolated from natural waters, soil or cyanide-containing wastes (DASH; GAUR; BALOMAJUMDER, 2009).

Several authors have previously reported the use of microbial strains to degrade cyanide (ANNACHHATRE; AMORNKAEW, 2000; BAXTER; CUMMINGS, 2006; DASH; GAUR; BALOMAJUMDER, 2009; KUYUCAK; AKCIL, 2013). This treatment can be performed with individual strains or in consortia (MEKUTO et al., 2013). Furthermore, several genera have been studied for cyanide degradation including *Pseudomonas* (WATANABE et al., 1998; HUERTAS et al., 2010; NWOKORO; DIBUA, 2014; LUQUE-ALMAGRO; MORENO-VIVIÁN; ROLDÁN, 2016; SINGH; BALOMAJUMDER, 2016), and *Bacillus* strains (SKOWRONSKI; STROBEL, 1969; WU et al., 2014; KANDASAMY et al., 2015; RAZANAMAHANDRY et al., 2016). These strains are able to lower cyanide concentrations by using it in their metabolism and in different temperatures, pH, initial concentrations, or carbon source conditions (MEKUTO et al., 2015; KARAMBA et al., 2016).

Cyanide biotreatment must thus be studied in order to understand cyanide-resistant populations and conditions that limit or enhance cyanide

biological degradation. Therefore, this study aims to isolate, characterize, and identify cyanide-degrading microorganisms from a cassava-processing industry, and assess their treatment potential in order to develop the needed knowledge for the improvement of cyanide treatment biotechnologies.

2 OBJECTIVES

The main objective of this study is the isolation, screening, and characterization of cyanide-degrading bacteria from a cassava-processing wastewater sample and the enhancing of free-cyanide degradation by the isolated strains.

In order to achieve this, this study aims to:

- Isolate, describe and identify native bacteria with the capacity to resist the presence of cyanide.
- Assess the potential for free-cyanide degradation of isolated strains
- Evaluate the susceptibility of isolated strains to pH and initial cyanide concentration conditions.
- Analyze the influence of the carbon source, temperature and agitation speed in cyanide removal of selected strains.

3 LITERATURE REVIEW

3.1 CYANIDE CHEMISTRY

Cyanide is a chemical group formed by a carbon and a nitrogen atom linked by a triple covalent bond. It is a monovalent ion that can be present in the form of hydrocyanic acid (HCN), free cyanide ion (CN^-), forming salts or metal complexes (ATSDR, 2006). Other chemical species associated with cyanide are cyanate (CNO^-), thiocyanate (SCN-), cyanogen chloride (CNCl) and nitriles.

Cyanides can be produced naturally in the environment by plants, fungus, algae, and bacteria (POULTON, 1990; LUQUE-ALMAGRO et al., 2005b). There's a natural non-toxic concentration of cyanide in some food including apple seeds, soy, cassava, and almonds and it is also present in the environment as a result of human activities including mining, electroplating, nylon production, and coke processing (MUDDER; BOTZ, 2004).

Cyanide chemical species can be divided into three classes when measuring it: total cyanide, WAD cyanide, and free cyanide. Free cyanide refers to CN^- and HCN dissolved in aqueous solution; WAD cyanide includes free cyanide and weak metal-cyanide complexes that include Ag, Cd, Cu, Hg, Ni, and Zn; total cyanide includes WAD cyanide and strong metal-cyanide complexes with Au, Co, and Fe (BOTZ; MUDDER; AKCIL, 2016). Metal-cyanide complexes are classified as weak or strong according to physicochemical criteria (JOHNSON, 2015). Environmentally, free cyanide and WAD cyanide are the most important species since they represent a higher environmental risk and are more toxic than strong metal complexes.

3.1.1 CYANIDE STABILITY

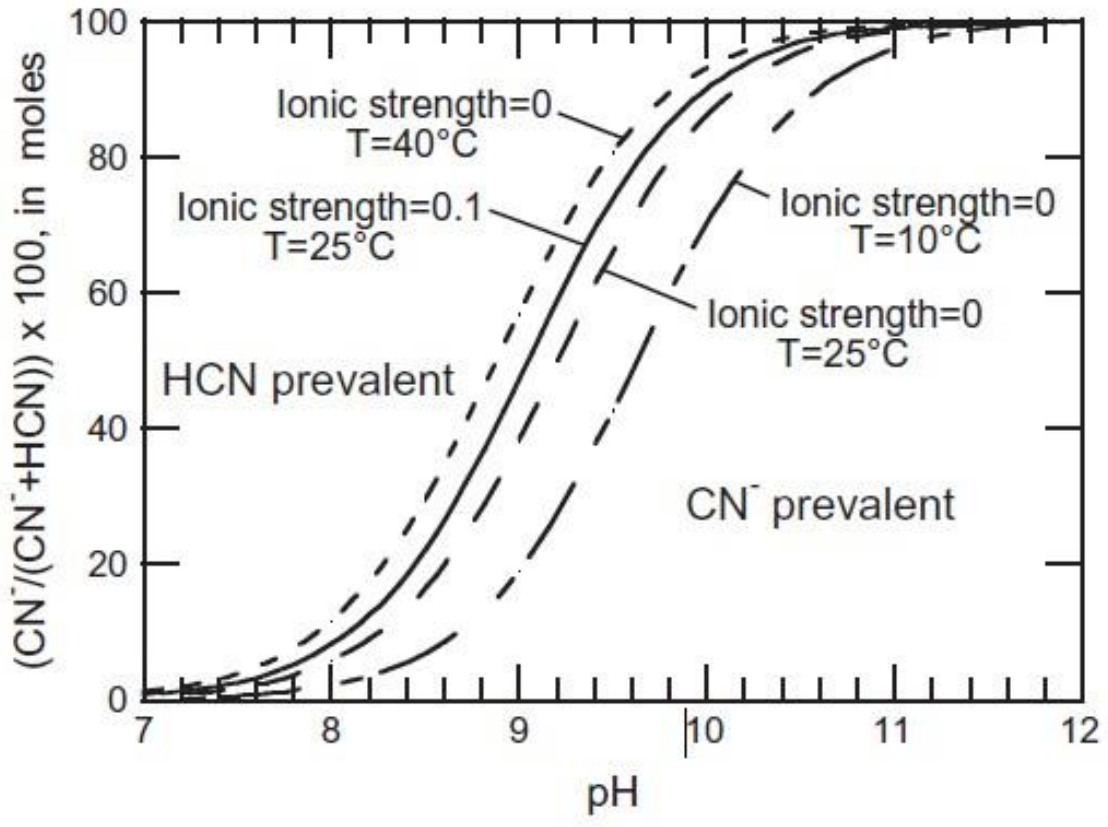
Free cyanide stability is dependent on pH with a pKa of 9.2 at 25 °C. Cyanide dissociation occurs according to Equation 1. However, this varies with ionic strength and temperature as shown in Figure 1.

$$CN^- + H^+ \leftrightarrow HCN_{(gas)} \qquad \text{Equation 1}$$

Meanwhile, stability changes when explaining cyanide complexes since these substances have a wide range of chemical and biological characteristics

(DASH; GAUR; BALOMAJUMDER, 2009). Some cyanide-metal complexes can even be stable at pH 7 such as the Ni-CN complex where free cyanide would be highly volatile (SNOEYINK; JENKINS, 1980).

Figure 1. Relative abundance of dissolved CN^- and HCN in cyanide solutions as a function of pH.



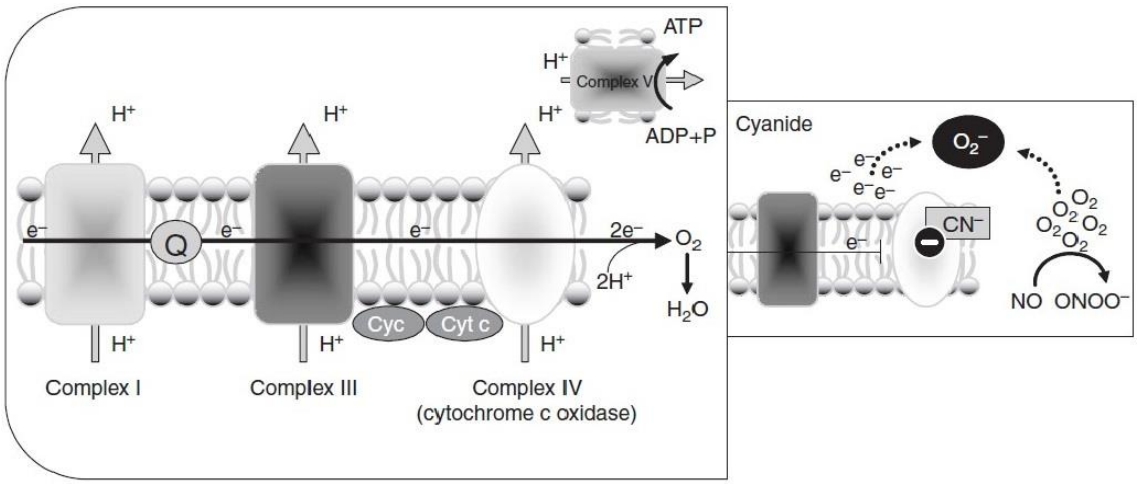
Source: (JOHNSON, 2015)

3.1.2 CYANIDE TOXICITY

The toxicity of cyanide compounds results from binding to the ferric ion in metalloenzymes, inhibiting cytochrome c oxidase in the mitochondrial electron transport chain (ETC) and preventing tissues from using sufficient oxygen causing histotoxic hypoxia throughout the body in aerobic organisms as shown in Figure 2 (HAMEL, 2011). This inhibition of cytochrome is irreversible so it causes permanent damage (BOUWMAN et al., 2006). As a result, cyanide causes a drop in the production of intracellular ATP preventing homeostasis cells, causing hard breathing, vomiting, headache, blood disorders, and enlarging the thyroid gland. Exposure to cyanide-containing solutions or wastes causes

damage in the brain, central nervous system, heart, and kidneys, and can produce coma and even death (ATSDR, 2006).

Figure 2. Electrons Transport Chain (ETC) in presence of cyanide.



Source: Amended from (BOUWMAN et al., 2006)

Because cellular respiration is common in all organisms, the presence of cyanide in the environment is harmful to all forms of living. Moreover, the presence of cyanide compounds in aquatic environments is an environmental risk as they have sub-lethal and lethal effects in concentrations as low as 0.01mg.L^{-1} CN^- (WILD, S.R. ; RUDD, T. ; NELLER, 1994). Meanwhile, anaerobic microorganisms are also inhibited by cyanide, especially methanogens; since cyanide inhibits the activity of essential metallo-proteins in these organisms (GIJZEN; BERNAL; FERRER, 2000).

Although free cyanide is known to be the most toxic form of cyanide, cyanide toxicity can vary when present in metal-complexes as shown in Table 1. For most complexes, the environmental significance stems from their tendency to release free cyanide by dissociation. Hence, strong metal-cyanide complexes are generally considered to have less environmental significance than weak complexes because they do not dissociate easily. However, the strong cyanide complexes of iron and cobalt undergo photodissociation, so the decomposition o these species can be catalyzed where solutions are exposed to sunlight (JOHNSON, 2015). Furthermore, the rate of dissociation is affected by factors such as the intensity of light, water temperature, pH, total dissolved solids and complex concentration (KUYUCAK; AKCIL, 2013). In general, metal complexes tend to increase the stability of cyanide and, therefore, decrease its toxicity.

Nevertheless, metal-cyanide does not eliminate the toxicity of cyanide and it thus is mandatory to be removed from effluents to prevent pollution prior to discharge to the environment (PATIL; PAKNIKAR, 2000).

Table 1. Cyanide species and their relative acute toxicity

Group	**Species**	**Intrinsic Toxicity**
Free Cyanide	CN^- HCN_{aq}	High
Weak Complexes	$Zn(CN)_4^{-2}$ $Ag(CN)_2^-$ $Cd(CN)_4^{-2}$ $Ni(CN)_4^{-2}$ $Cu(CN)_3^{-2}$ $Cr(CN)_6^{-3}$ $Mn(CN)_6^{-3}$	Intermediate
Strong Complexes	$Fe(CN)_6^{-3}$ $Fe(CN)_6^{-4}$ $Co(CN)_6^{-4}$ $Au(CN)_2^-$	Low
Cyanide-related species	CNO^- SCN^- C_2N_2 CNCl	Low Low High High

Source: (JOHNSON, 2015)

Because strong cyanide complexes are very chemically stable, WAD cyanide and free-cyanide are the most important species for the environment (JOHNSON, 2015). The release of these cyanide compounds can result in severe toxic effects in ecosystems and therefore, monitoring their concentration in wastewaters is an important parameter to assess the quality of cyanide-containing effluents.

3.2 LEGISLATION

In order to protect the environment, many countries have established cyanide limits in natural environments and industrial discharges. In the USA, the limit established by EPA is 0.2mg.L^{-1} of total cyanide for aquatic environments and 0.05mg.L^{-1} of total cyanide for drinking water (ENVIRONMENTAL PROTECTION AGENCY, 2010). Meanwhile, European countries as Germany and Switzerland, have limits of 0.01mg.L^{-1} for surface waters and 0.5mg.L^{-1} for sewers. In Latin America, Mexico has established a limit of 0.2mg.L^{-1} for cyanide

dispositions and Brazil has a 1mg.L^{-1} total cyanide limit for effluent discharge (CONSELHO NACIONAL DO MEIO AMBIENTE-CONAMA, 2011).

3.3 CYANIDE APLICATIONS

Cyanide is used in several industries including electroplating, synthetic fiber production, coal processing, jewelry industry, fire retardants, cosmetics, pesticide production, and metallurgic processes of gold and silver (MUDDER; BOTZ, 2004; HUERTAS et al., 2010).

The most important cyanide application is in mining, which consumes nearly 13% of cyanide salts produced in the world. Cyanide salts are used in the leaching of gold and silver from ores to form cyanide complexes $[Ag(CN)_2]^-$ and $[Au(CN)_2]^-$ extracting these precious metals from ores and making them soluble in water in alkaline conditions (KUYUCAK; AKCIL, 2013). Cyanidation is a widely-used process to obtain gold due to a combination of availability, effectiveness, economics, and risk (MUDDER; BOTZ, 2004); and since it allows to recover up to 80 or 90% of gold present in gold (VEIGA et al., 2014) by the complexation of gold according to the Elsner's equation (Equation 2):

$$4Au + 8NaCN + O_2 + 2H_2O \rightarrow 4Na[Au(CN)_2] + 4NaOH \qquad \text{Equation 2}$$

Similarly, cyanide properties to form metal complexes are used in other industries. In electroplating processes, electric currents are used to form a thin metal coating on an object to modify material properties including anti-corrosion coating, abrasion resistance, and aesthetic qualities. Electroplating baths often use cyanide electrolytes with cyanide salts as a major bath constituent as it facilitates anode corrosion, maintains constant metal ion levels, and contributes to conductivity (DINI; SNYDER, 2010)

Annually, about 1.1million tons of HCN are produced worldwide (MUDDER; BOTZ, 2004). Gold processing and electroplating industries use together approximately 20% of total cyanide produced in the world and, therefore, are responsible for the emission of cyanide-containing effluents and solid wastes (MPC, 2000; AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY, 2006).

3.3.1 CYANIDE-CONTAINING EFFLUENTS

The consumption of cyanide salts or metal-cyanide salts in industries results in the production of effluents and solid wastes that also contain cyanide. Because of this, and due to cyanide intrinsic toxicity, discharging cyanide-containing effluents to the environment represents an environmental risk.

Although, cyanide-containing effluents do not have a unique composition and will present a wide variety of characteristics depending on the industry, the size of the operation, the processes originating the waste, and the efficiency of procedures. In general, cyanide-containing effluents can present total-cyanide concentrations from 0.01 to 30000 $mg.L^{-1}$ (DASH; GAUR; BALOMAJUMDER, 2009) and they can present different proportions between total cyanide and free-cyanide proportions as shown in Table 2.

Table 2. Concentration of cyanide emitted from various industries

Industry	**Total CN (mg.L-1)**	**% free-CN**
Coke-oven wastewater	58,8	1.1
Coke plant	0.1 - 150	1.17
Electroplating plant	0.03 - 60	26
Electroplating process	2100	-
Plating rinse	4 - 260	-
Plating Bath	4000 - 60000	-
Steel industry	3 - 400	-
Chemical industry	10 - 60	0.3
Gold ore extraction	18 - 25	-
Silver electroplating	5	-
Oil refinary	0 – 2.5	0
Starch production from cassava	3 – 5	-
Cassava mill wastewater	19 – 86	-

Source: Adapted from PATIL; PAKNIKAR, 1999; MARDER; BERNARDES; ZOPPAS FERREIRA, 2004; COLIN et al., 2007; DASH; GAUR; BALOMAJUMDER, 2009; KAEWKANNETRA et al., 2009

Cyanide-containing effluents can also present other pollutants such as ammonia, suspended solids and other metals that represent important variables when choosing a proper treatment method. For example, mining industries will produce effluents that contain suspended solids from the treated ore but cyanide concentrations as low as 50ppm. Meanwhile, electroplating industries will

generate effluents low in organic matter with higher total-cyanide concentrations. Even when cyanide concentrations are not critical when compared to other pollutants in effluents, the presence of cyanide can affect conventional Wastewater Treatment Plants (WWT) when carelessly discharged to sewage systems since they usually operate under biological systems that do not contain cyanide-resistant bacteria (WILD, S.R. ; RUDD, T. ; NELLER, 1994).

3.4 CASSAVA-PROCESSING INDUSTRY

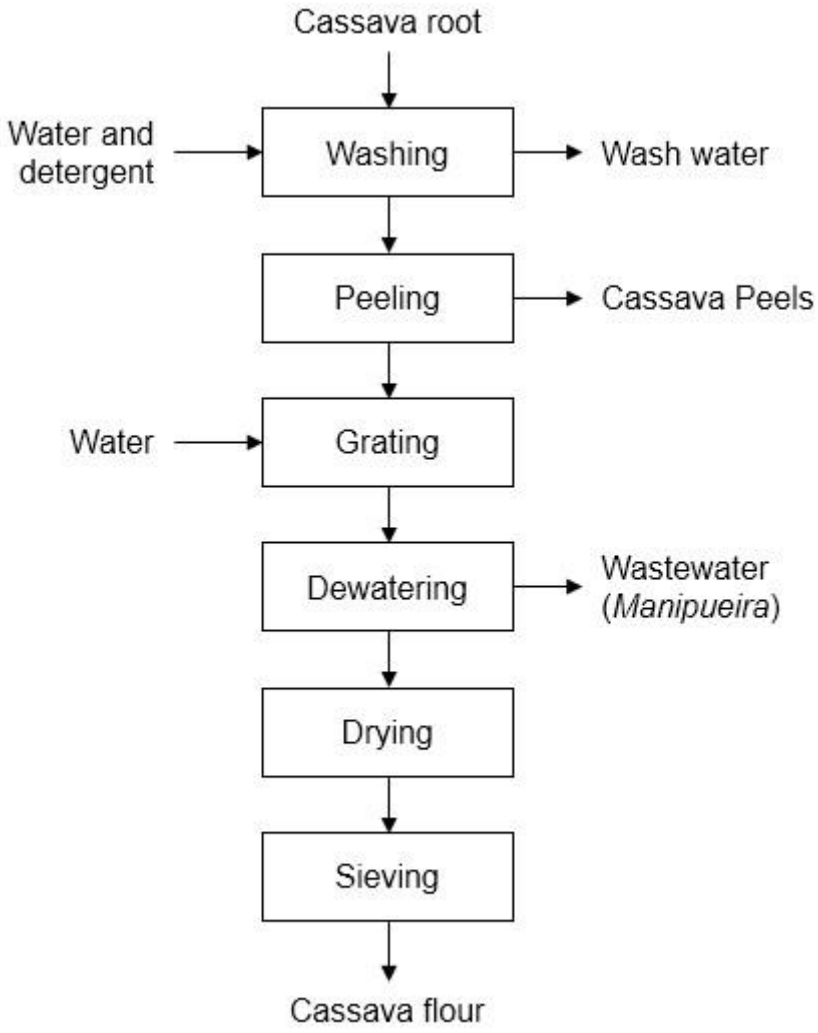
Cassava (*Manihot esculenta*) is a plant original from Latin America, cultivated for the last 4000 years and spread around the world due to its ability to grow in areas with acid and low-fertility soils, and low or unpredictable rainfall (AKINPELU, A.O. L. E.F. AMAMGBO, A.O. OLOJEDE, 2011). Cassava roots are a major source of carbohydrates since it can present up to 91% of the dry weight in the whole root (ALVES; AUGUSTO; ALVES, 2002). Cassava thus is the fourth most important source of dietary energy in the tropics and the 9th world-wide; and two-thirds of its global production is located in Nigeria, Brazil, Thailand, DR Congo and Indonesia (BOKANGA, 1999).

Brazil is the second-largest cassava producer in the world, achieving 25million tons of cassava produced in 2005 (VILPOUX, 2008). In Brazil, cassava is widely consumed across the country and cassava flour is the most common product from cassava industrial use although most of the production is only locally consumed (SOUZA et al., 2008). Cassava consumption can be different according to plant variety and cultural costumes. There are two types of cassava plants: “Sweet” cassava and “bitter” cassava. The difference between these types is their cyanide content and their use. Sweet cassava is commonly eaten raw in Africa or cooked in Brazil (BALAGOPALAN, 2002), while bitter cassava is commonly used in industrial applications since these processes tend to reduce the cyanide content in products (CHISTÉ et al., 2010).

Cassava flour (“*farinha*”) is the product of cassava roots after being cleaned, peeled, grated, dewatered by a press and dried or roasted in slow fire (BALAGOPALAN, 2002). In most cases, bitter cassava is used for cassava flour production. During cassava flour production, industries commonly follow the process showed in Figure 3. First, cassava roots are received and weight after

being sieved for dirt removal. Then, they enter in a washing unit where water and detergent are used to remove residual dirt. Cassava roots are then peeled automatically or manually and peels and plant wastes (stems and leaves) are discarded. Then, cassava is fed to a mechanical grater to transform roots into a fine mash. Afterward, the wet pulp is squeezed in a pressing machine to extract the extra water; this step is known as the dewatering process. From the dewatering process is produced the cassava-processing wastewater, also known in Brazil as *manipueira*, which contains residual pulp and soluble components of cassava. Manipueira is often kept separately from washing wastewater since they are two effluents with different composition and, therefore, treatment needs. After the press, cassava pulp goes through a drying process that can be natural (sun-dried), by airflow, in an oven or in a rotary dryer. Finally, cassava flour if sieved and packed for commercialization. Some industries can also include a fermentation unit before the dewatering process to produce starch, which is a higher quality product than flour.

Figure 3. Process flow chart for cassava flour production



Source: Personal Collection

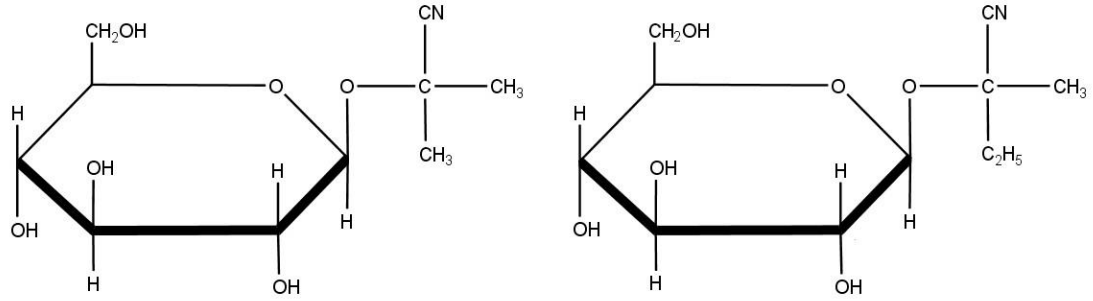
3.4.1 CYANOGENESIS IN CASSAVA

Cyanide can be produced naturally by plants, fungus, algae, and bacteria (ATSDR, 2006). Plants that produce cyanide are called “cyanogenic plants”; these include almonds, bamboo, cassava, cashew, olives, potatoes, soybeans, and lentils. The most common source of cyanide poisoning from natural sources due to food intake is cassava, especially in equatorial countries of the world (MUDDER; BOTZ, 2004).

During cassava digestion or industrial processing, cyanide is liberated from cassava pulp due to the presence of cyanogenic glycosides, linamarin and lotaustralin (Figure 4). Linamarin is the most common cyanogenic glycoside in

cassava pulp, constituting the 93% of the total cyanogenic glycoside present in cassava tissues (MCMAHON; WHITE; SAY, 1995). Cyanogenic glycosides are distributed in all the cassava plant; however, the presence of these molecules is higher in bitter varieties, especially in leaves, roots, and stems of the plant, presenting total cyanide concentrations higher than 100mgHCN.kg^{-1} of dry root in bitter varieties (MCMAHON; WHITE; SAY, 1995; ALVES; AUGUSTO; ALVES, 2002; MUDDER; BOTZ, 2004; CHISTÉ et al., 2010).

Figure 4. Cyanide glycosides present in cassava



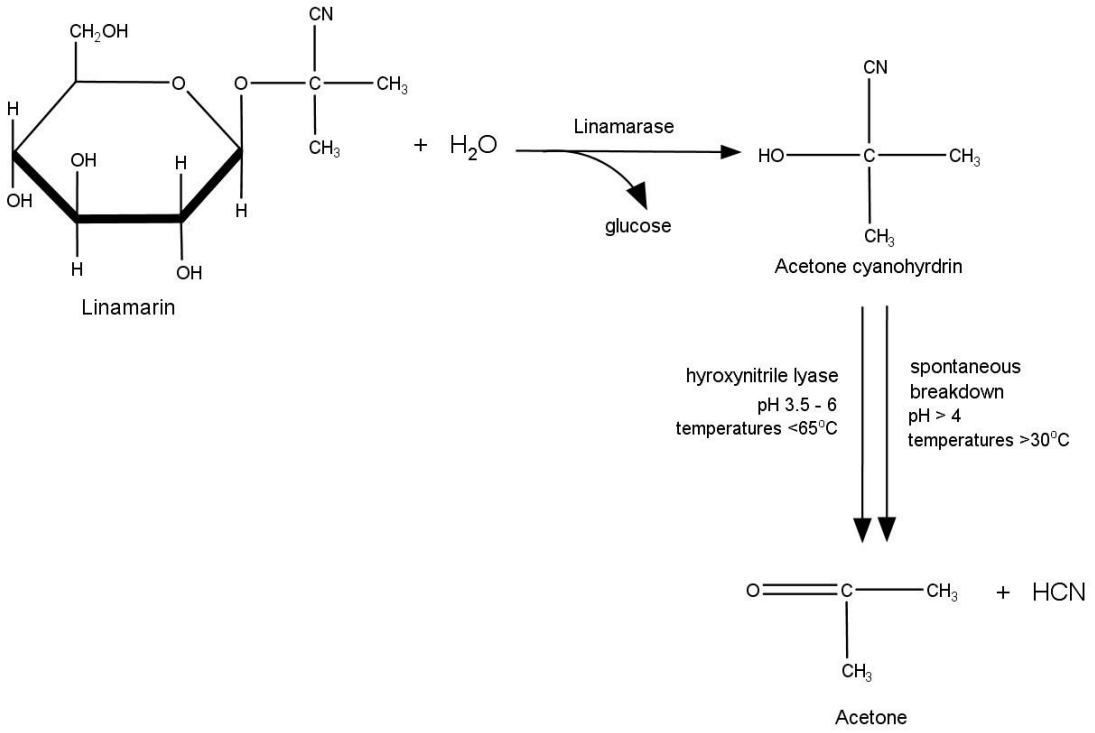
Source: Personal collection

Cyanogenesis from linamarin (Figure 5) is a two-step process that begins with the hydrolyzation of the molecule by a β-glucosidase enzyme (linamarase). Linamarin and linamarase are both present in cassava cells but are located in different parts of the cell, so they are together only after the pulp-grating process or during digestion when the plant tissue is broken. The reaction produces α-hydroxynitriles (Acetone cyanohydrin) and liberates glucose. The second step is the decomposition of acetone cyanohydrin in acetone and cyanide, which can occur spontaneously or can be catalyzed by a hydroxynitrile lyase (HNL) enzyme. Therefore, the enzymatic activity of linamarase is the limiting factor in the transformation of linamarin to free cyanide (MCMAHON; WHITE; SAY, 1995; CEREDA; MATTOS, 1996).

However, linamarase can also be produced by microbes. Yeast and lactic acid bacteria (LAB) are associated to the production of linamarase including bacteria belonging to the *Lactobacillus*, *Streptococcus, Corynebacterium, Bacillus,* and *Pseudomonas* genera and have been associated to the

detoxification of cyanogenic glycosides during cassava-processing (BEHERA; RAY, 2017).

Figure 5. Cyanogenesis from linamarin



Source: Adapted from MCMAHON; WHITE; SAY, 1995

3.4.2 WASTE MANAGEMENT IN CASSAVA PROCESSING

During cassava flour production, solid and liquid waste are generated. Solid wastes are mainly cassava peel, which represents between 2 and 5% of the weight of the root (BALAGOPALAN, 2002). Cassava peel often includes residual pulp that could not be extracted, contains fibrous material and since it is removed after the washing process, it is always wet. This solid waste can thus be used as animal feed or used in animal feed production (DE CARVALHO et al., 2018).

Because of water consumption during cassava flour production during the washing and the grating processes, cassava-processing wastewater (CPW) is the most significant waste in quantity during cassava industrial processing (BALAGOPALAN, 2002). Cassava-processing wastewater, which is the mixture of cassava water and process water, has a very high eutrophication potential due

to the presence of carbon (C), nitrogen (N), and phosphorus (P) concentrations (DE CARVALHO et al., 2018). CPW also presents cyanide in toxic concentrations and a Chemical Oxygen Demand (COD) above the limit allowed by legislation, making mandatory its treatment as industrial effluents. Because of this, some technological alternatives for CPW have been previously studied including using it as animal manure (ALMEIDA et al., 2009), hydrogen production (KHONGKLIANG et al., 2017; MADEIRA et al., 2017), enzyme production (JIN et al., 1999), algae production (DE CARVALHO et al., 2018), and biogas production (JIRAPRASERTWONG; MAITRIWONG; CHAVADEJ, 2019).

3.5 TREATMENT TECHNOLOGIES FOR CYANIDE-CONTAINING WASTES AND WASTEWATERS

During the production of goods and services, unwanted wastewaters and solid wastes are generated as a result of the inefficiency of processes or impurities in materials. Depending on their composition, solid wastes and wastewaters might represent a risk for environmental safety and public health, especially when they have toxic compounds as cyanide in their composition. Industrial effluents thus receive special attention due to their potential to pollute underground water and their potential to affect processes at wastewater treatment plants. Therefore, industrial effluents are commonly treated within the company's facilities to comply with regulations to enter the sewage systems or to be discharged in natural waters.

In order to solve this problem, it is mandatory to know the key characteristics of effluents to be treated including organic matter presence, solids content and toxic substances concentration (LIMA et al., 2007). The polluting load of effluents can be reduced by natural, chemical, physical and biological processes that aim to achieve a waste quality that does not represent an environmental or public health risk.

In order to achieve safe concentrations according to legislation, cyanide has to be treated from effluents and wastes prior to discharge to the environment. The common cyanide treatment methods include natural degradation in tailings ponds, chemical and biological degradation processes where volatilization, photodecomposition, chemical oxidation, microbial oxidation, chemical

precipitation, hydrolysis or precipitation on solids occur transforming cyanide into less toxic products (DASH; GAUR; BALOMAJUMDER, 2009).

Optimal cyanide treatment should be able to degrade the most toxic species of cyanide together or be able to degrade free cyanide in the presence of other pollutants including metals. Furthermore, the method has to be fast, easy and cheap; thus, it has to be designed to meet local needs for every industry and location (HINTON; VEIGA; VEIGA, 2003) and should not produce undesirable by-products during the process.

3.5.1 NATURAL DEGRADATION

Natural degradation of cyanide is based on the processes of volatilization, oxidation, hydrolysis, photodecomposition, and precipitation that occur naturally without human intervention. In order to achieve this, cyanide-containing wastes are exposed to the environment in large tailing ponds where cyanide degradation occurs influenced by pH, temperature, sunlight, bacteria present and cyanide concentration (KUYUCAK; AKCIL, 2013). Tailing ponds are thus designed maximizing the surface area in order for the waste to interact chemically with the atmosphere (BOTZ; MUDDER; AKCIL, 2016).

Cyanide attenuation occurs when the natural uptake of carbon dioxide from the air reduces the pH level in the pond, forming HCN and thus causing cyanide volatilization. At the same time, cyanide can also be oxidized by dissolved oxygen in the pond transforming cyanide in cyanate and then to ammonia and carbon following Equation 3 and Equation 4 (YOUNG; JORDAN, 1995).

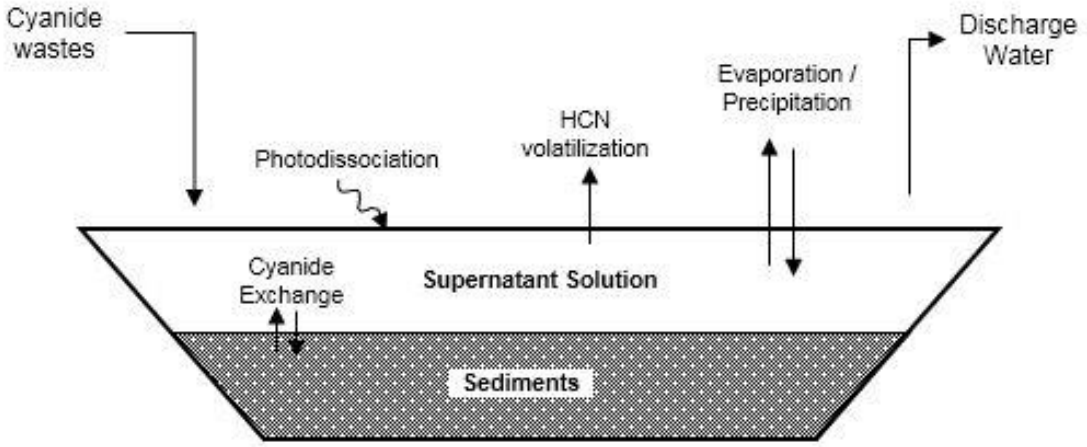
$$CN^- + \frac{1}{2}O_2 \rightarrow OCN^- \qquad \text{Equation 3}$$

$$OCN^- + H_2O \rightarrow {HCO_3}^- + {NH_4}^+ + OH^- \qquad \text{Equation 4}$$

The processes for cyanide natural attenuation that occur in a tailing pond can take place in the solid accumulation zone or in the liquid accumulate (Figure 6). The main route for cyanide loss is HCN volatilization from the liquid surface, accounting for up to 90% of cyanide removal, which is also enhanced by the

equilibrium pH in tailing ponds between 9 and 10 (BOTZ; MUDDER; AKCIL, 2016). Because of this, when using a natural degradation process in cyanide treating, temperature and aeration are the most important variables to be controlled. Furthermore, the operation costs of using tailing ponds are much lower than any other technology and it can produce acceptable cyanide concentrations in effluents for discharge and when not, it can be used as an intermediate step prior to active treatment (KUYUCAK; AKCIL, 2013).

Figure 6. Cyanide-degrading processes in tailing ponds.



Source: Amended from BOTZ; MUDDER; AKCIL, 2016

3.5.2 PHYSICAL AND CHEMICAL METHODS

When cyanide-containing effluents present chemical characteristics that don't allow natural degradation or there is no space available for tailing pond construction to attend the industry effluent generation, active processes need to be applied prior to discharge to the environment to achieve non-dangerous concentrations of cyanide. The most commonly adopted methods for cyanide treatment are the chemical oxidation techniques including alkaline-chlorination-oxidation, hydrogen peroxide oxidation, and SO_2/Air process, which are described below.

3.5.2.1 ALKALINE CHLORINATION

The alkaline chlorination process is the most widely used treatment for cyanide-containing effluents and has been applied since de commercial

development of cyanide leaching of gold (YOUNG; JORDAN, 1995). In this process, cyanide is destroyed by a solution with chlorine in a two-step process, first transforming it to CNCl and then to cyanate as shown in Equation 5 and Equation 6 (AKCIL, 2003):

$$\mathrm{Cl_2 + CN^- \rightarrow CNCl + Cl^-} \qquad \text{Equation 5}$$

$$\mathrm{CNCl + H_2O \rightarrow OCN^- + Cl^- + 2H^+} \qquad \text{Equation 6}$$

In the presence of chlorine in excess, cyanate can be further oxidized to ammonia and later, transformed in nitrogen a carbon dioxide implying greater reagent consumption. In order to achieve this, chlorine can be added as a gas or as a salt in the form of NaClO or $Ca(ClO)_2$ (BOTZ; MUDDER; AKCIL, 2016). However, chlorine consumption and pH control highly increase the cost of the process (KUYUCAK; AKCIL, 2013).

Although efficient in the removal of free cyanide and WAD cyanide, alkaline chlorination is not effective in the treatment of ferrocyanide complexes and other strong cyanide complexes (YOUNG; JORDAN, 1995; JOHNSON, 2015). Furthermore, the alkaline chlorination process uses expensive reagents; requires carefully condition control; and can form organ-chlorinated compounds, chlorocyanic gases and can liberate chloride and hypochlorite to the environment, which are both toxic (YOUNG; JORDAN, 1995).

3.5.2.2 HYDROGEN PEROXIDE OXIDATION

Another important and widespread treatment for cyanide-containing wastes is oxidation using hydrogen peroxide (H_2O_2) in presence of a transition metal such as copper (Cu) that acts like a catalyst (AKCIL, 2003; KUYUCAK; AKCIL, 2013; BOTZ; MUDDER; AKCIL, 2016). During this process, cyanide solutions are treated when reacting to hydrogen peroxide according to Equation 7 and Equation 8.

$$H_2O_2 + CN^- \xrightarrow{Cu^{+2}\ Catalyst} OCN^- + H_2O \qquad \text{Equation 7}$$

$$OCN^- + 2H_2O \rightarrow CO_3^{2-} + NH_4^+ \qquad \text{Equation 8}$$

The hydrogen peroxide process can treat effectively cyanide present in liquid solutions and can also be applied in the decontamination of slurry although causing a high consumption of hydrogen peroxide (AKCIL, 2003). Some of the main advantages of using this treatment are that it can treat cyanide in a wide range of pH; it can remove cyanide-related compounds during treatment, and reagent decomposes to waste and oxygen without leaving other toxic compounds (DASH; GAUR; BALOMAJUMDER, 2009; BOTZ; MUDDER; AKCIL, 2016). Nevertheless, the process doesn't lower the levels of thiocyanate, and H_2O_2 causes difficulties in handling because of its expensive and hazardous characteristics (KUYUCAK; AKCIL, 2013). Moreover, the amount of ammonia produced during treatment can still cause damage to the environment when discharged to natural waters (DASH; GAUR; BALOMAJUMDER, 2009).

3.5.2.3 SULFUR DIOXIDE/AIR PROCESS

This process is patented and it is also known as the INCO process (GOODWIN, 1989). It is similar to the process using H_2O_2, but it uses a mixture of SO_2 and air to oxidize cyanide in the presence of copper as a catalyst according to the Equation 9 (BOTZ; MUDDER; AKCIL, 2016):

$$SO_2 + O_2 + H_2O + CN^- \xrightarrow{Cu^{+2}\ Catalyst} OCN^- + SO_4^{2-} + 2H^+ \qquad \text{Equation 9}$$

Similarly to the hydrogen peroxide process, the INCO process does not remove thiocyanate but removes 6other cyanide related compounds including metal complexes precipitating copper-iron-cyanide complex (BOTZ; MUDDER; AKCIL, 2016). Moreover, INCO process can effectively treat slurries and reagent costs are very inexpensive compared to alkaline chlorination or hydrogen peroxide oxidations; however, some reagents may need license or royalty payments and the process can add sulfated to treated waters (DASH; GAUR; BALOMAJUMDER, 2009).

3.5.3 APPLIED BIOTECHNOLOGY FOR CYANIDE TREATMENT

When pollutants are organic, biotreatment is often the best solution due to its relative simplicity and efficiency at low costs (HATZINGER; KELSEY, 2005; LIMA et al., 2007). Biodegradation is the partial or complete breakdown of organic

molecules in smaller components by the action of living organisms (CRAWFORD, 2011). This process results in less complex molecules than can be more or less toxic than original pollutants or they can be “mineralized” when transformed completely into inorganic non-toxic products thus reducing the risk associated with the presence of a particular xenobiotic (HATZINGER; KELSEY, 2005).

Generally, microorganisms use their metabolic pathways to obtain nutrients and energy from organic molecules for their vital functions (HATZINGER; KELSEY, 2005). This metabolism can be dependent on the presence of oxygen (aerobic process) or the absence of oxygen (anaerobic process) and transform pollutants by enzymes into metabolites that may be used in other cellular metabolic pathways (JØRGENSEN, 2008).

Microorganisms, especially bacteria, have evolved through time due to extreme stress conditions and competition for limited resources. This has led to the development of metabolic capabilities to degrade almost every organic molecule including pollutants present in wastes as lignin, hydrocarbons, pesticides, pharmaceutical substances, and other xenobiotics (HATZINGER; KELSEY, 2005; CRAWFORD, 2011). Because of that, bioremediation can use microorganisms to clean unwanted organic pollutants from wastes and wastewaters by transforming them into simpler, smaller and less toxic molecules.

However, biodegradation processes are influenced by the concentration of nutrients, the presence or absence of oxygen, the pH and the chemical and physical characteristics of the waste to be treated (HATZINGER; KELSEY, 2005). Hence, understanding these processes requires scientific research under different approaches including analytical chemistry, enzymology, genomics, and proteomics that contribute to the determination of how and under what conditions pollutants are degraded (CRAWFORD, 2011) aiming to the application of biotechnology in the reduction of pollution.

Instead of the chemical and natural processes previously explained, biotreatment is an alternative technology for cyanide decontamination. Native biological populations in wastes are adapted to pollutants concentrations and promote growth while transforming cyanide and other toxic substances into less toxic metabolites (DOBLE; KUMAR, 2005). Because it uses microorganisms

naturally present in wastewaters, the biological treatment of cyanide is often offered as a cheaper and cleaner alternative and with that, a more sustainable technology for the treatment of cyanide-containing effluents (MUDDER; BOTZ, 2004).

Cyanide can be treated by organisms in both aerobic and anaerobic conditions (ANNACHHATRE; AMORNKAEW, 2000; EBBS, 2004) and in active and passive systems (KUYUCAK; AKCIL, 2013). Although cyanide degradation by plants (phytoremediation) has been demonstrated (AKSU et al., 1999; TRAPP; MORTEN; CHRISTIANSEN, 2001), only microbial degradation of cyanide will be the scope of this study since microorganisms have adapted to the presence of cyanide and other pollutants and their use can couple the detoxification of cyanide with denitrification of resulting ammonia to produce effluents with weaker environmental impacts when discharged (BAXTER; CUMMINGS, 2006).

Even though the biological degradation of chemical pollutants is considered a cheaper technology and more environment-friendly, the biological treatment of cyanide also presents disadvantages compared to traditional chemical processes. Its susceptibility to environmental conditions may inhibit the microbial activity and affect the quality of the treatment (BAXTER; CUMMINGS, 2006; DASH; GAUR; BALOMAJUMDER, 2009). Furthermore, processes tend to be very site-specific and its application tends to be difficult to adapt to different site operations, especially to cold environmental temperatures than can stop biological metabolism (KUYUCAK; AKCIL, 2013).

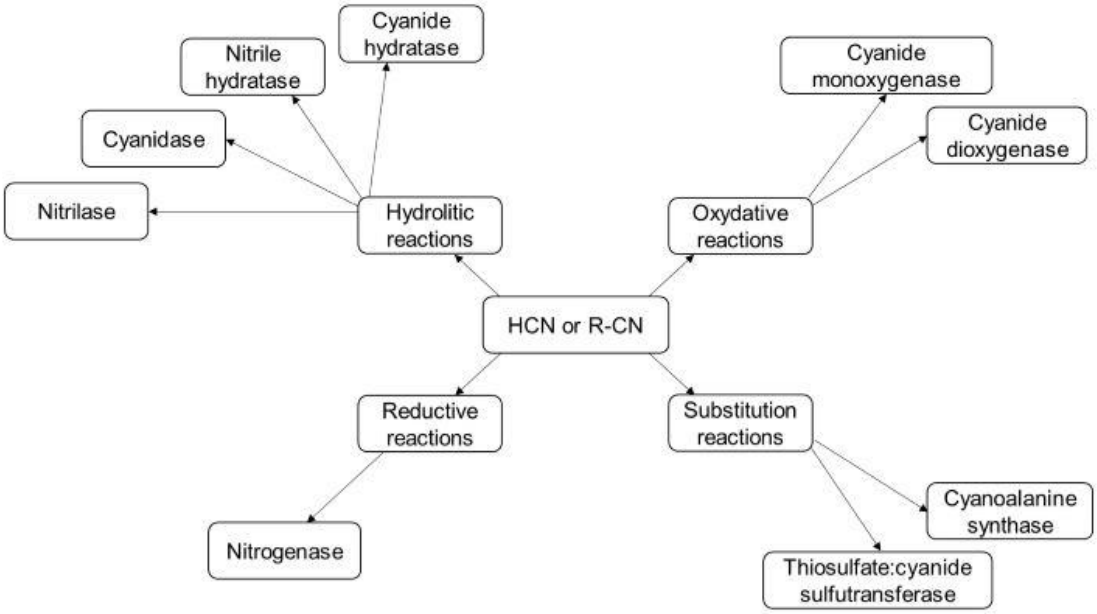
During microbial degradation, bacteria, algae, and fungi transform cyanide compounds in cyanate, formic acid, bicarbonate, ammonia, and other metabolites according to metabolic pathways (EBBS, 2004). In general, biological transformation involves cyanide degradation to form amino acids, thiocyanate, β-cyanoalanine and vitamins that are later mineralized to carbon dioxide and ammonia (GUPTA; BALOMAJUMDER; AGARWAL, 2010)

3.5.3.1 METABOLIC PATHWAYS FOR CYANIDE DEGRADATION

Cyanide compounds can be metabolized by microorganisms using enzymatic routes. Four types of enzymatic routes can metabolize cyanide

including hydrolytic, oxidative, reductive, and substitution/synthesis biochemical reactions catalyzed by enzymes shown in Figure 7 (DASH; GAUR; BALOMAJUMDER, 2009). Sometimes, organisms can use more than one pathway at the same time for cyanide degradation (GUPTA; BALOMAJUMDER; AGARWAL, 2010). This process can hence result in the production of inorganic nutrients, energy or biomass, or cyanide can be oxidized by cometabolism through metabolic pathways that are not specific for cyanide (HATZINGER; KELSEY, 2005). The enzymatic pathway used to degrade cyanide for each organism depends on the mechanism of tolerance in microbes, the presence of essential substrates and the presence of metals that could act as metabolic inhibitors (GUPTA; BALOMAJUMDER; AGARWAL, 2010).

Figure 7. Enzymatic pathways for cyanide degradation.



Source: Personal collection

Hydrolytic pathways can be catalyzed by cyanide hydratase, cyanidase, nitrile hydratase or nitrilase enzymes (BAXTER; CUMMINGS, 2006). The first two, act on HCN and the other two, on nitriles (R-CN) (DASH; GAUR; BALOMAJUMDER, 2009). Cyanide hydratase reacts to produce formamide; cyanidase produces formate and ammonia; and nitrilases and nitrile hydratases convert nitriles to the corresponding acid or amide, respectively (EBBS, 2004). This pathway results in the eventual conversion of cyanide in CO_2 and NH_3.

Oxidative reactions are catalyzed by cyanide monoxygenase and cyanide dioxygenase enzymes (DASH; GAUR; BALOMAJUMDER, 2009). Cyanide monoxygenase degrades cyanide to cyanate which is then transformed into carbon dioxide and ammonia by cyanase; on the other hand, cyanide dioxygenase converts cyanide directly into ammonia and carbon dioxide (EBBS, 2004). Along with the consumption of cyanide, this pathway requires NADPH and an extra carbon source to catalyze cyanide degradation (GUPTA; BALOMAJUMDER; AGARWAL, 2010).

Reductive pathways are usually considered to occur under anaerobic conditions (GUPTA; BALOMAJUMDER; AGARWAL, 2010). Nitrogenase activity has been showed to increase activity when in the presence of cyanide in resting cell cultures producing methane and ammonia (EBBS, 2004). The process requires high consumption of chemical energy released from the hydrolysis of ATP, and Mo and Fe proteins (GUPTA; BALOMAJUMDER; AGARWAL, 2010). Reductive enzymes as nitrogenase can be inhibited by oxygen, the ammonium ion and amino acids in high concentrations (KAO et al., 2003).

In Substitution/synthesis metabolic pathways, cyanide can be converted in β-cyanoalanine or α-aminonitrile (RAYBUCK, 1992). The consumption of cyanide by these pathways involves cyanide assimilation and promotion of growth of the microorganisms using cyanide as a nitrogen source to synthesize aminoacids that would later be used in the production of proteins (GUPTA; BALOMAJUMDER; AGARWAL, 2010). Substitution/synthesis pathways can be catalyzed by β-cyanoalanine synthase, γ-Cyano-α-aminobutyric acid synthase, or thiosulfate:cyanide sulfurtransferase. During these processes, NH_3 can be released and there is no direct requirement for O_2, NADPH, nor CO_2 is released (DASH; GAUR; BALOMAJUMDER, 2009). The use of microorganisms that combine nitrilase activity with β-cyanoalanine synthase can be an optimal biodegradation process since it combines reaction for cyanide assimilation and detoxification that are essential for the system to be maintained without the inclusion of additional organisms (EBBS, 2004; GUPTA; BALOMAJUMDER; AGARWAL, 2010).

Table 3 shows the chemical reactions for every enzyme previously described. Hence, it allows identifying the substrates and products of each enzymatic pathway for cyanide degradation.

Table 3. Enzymatic reactions for cyanide degradation

Reaction Type	Enzyme	Reaction
Hydrolytic Reactions	Cyanide hydratase	$HCN + H_2O \rightarrow HCONH_2$
	Nitrile hydratase	$R\text{-}CN + H_2O \rightarrow R\text{-}CONH_2$
	Cyanidase	$HCN + 2H_2O \rightarrow HCOOH$
	Nitrilase	$R\text{-}CN + 2H_2O \rightarrow R\text{-}COOH$
Oxidative Reactions	Cyanide monoxygenase	$HCN + O_2 + H^+ + NAD(P) \rightarrow HCON + NAD(P)^+ + H_2O$
	Cyanide dioxygenase	$HCN + O_2 + H^+ + NAD(P)H \rightarrow CO_2 + NH_3 + NAD(P)^+$
Reductive Reactions	Nitrogenase	$HCN + 2H^+ + 2e^- \rightarrow CH_2{=}NH + H_2O \rightarrow CH_2{=}O$ $CH_2{=}NH + 2H^+ + 2e^- \rightarrow CH_3\text{-}NH + 2H^+ + 2e^- \rightarrow CH_4 + NH_3$
Synthesis/ Substitution reactions	Cyanoalanine synthase	$Cysteine + CN^- \rightarrow \beta\text{-}cyanoalanine + H_2S$
	Thiosulfate-cyanide sulfurtransferase	$CN^- + S_2O_3 \rightarrow SCN^- + SO_3^{2-}$

Source: Data from RAYBUCK, 1992; EBBS, 2004; GUPTA; BALOMAJUMDER; AGARWAL, 2010

3.5.3.2 CYANIDE-DEGRADING SPECIES

The degradation of cyanide has been reported in several microorganisms including fungi, algae, and bacteria that are able to grow in medium containing cyanide, that use cyanide as a whole nitrogen source or that decomposes cyanide in presence of other nutrients. Some of the reported microorganisms that are able to degrade cyanide are listed in Table 4. However, not all cyanide-degrading microorganisms can be detected by culture-based methods,

so it is assumed the existence of other microorganisms that contribute to cyanide degradation in the environment (MEKUTO; NTWAMPE; AKCIL, 2016).

In the treatment of cyanide, microorganisms can transform cyanide compounds through their metabolic processes. This occurs most often from nitriles or free cyanide; however, metal-complexed cyanide has also been reported to be attenuated biologically, but in most studies, it is difficult to determine whether the attenuation was by direct utilization of the complex or by utilization of free cyanide released by complex dissociation (JOHNSON, 2015).

Table 4. Cyanide-degrading species in the literature

Microorganism	**Treated pollutant**	**Concentration**	**pH**	**Temp (ºC)**
Pseudomonas sp.	WAD cyanide	100-400mg.L^{-1}	9.2-11.4	30
Pseudomonas fluorescens	Ferrous (II) cyanide complex	100mg.L^{-1}	5	25
Furasium solani	Potassium Cyanide	0.5-0.8mM	9.2-10.7	30
Pseudomonas fluorescens	Fe(II)-cyanide	100mg.L^{-1}	5	25
Klebsiella oxytoca	Potassium Cyanide	0.58mM	7	30
Pseudomonas putida	Sodium Cyanide	100-400mg.L^{-1}	6.7	25
Stemphilium loti	Potassium Cyanide	20µM	6.5-7.5	25
Bacillus pumilus	Potassium Cyanide	2.5M	8.5-9	40
Trichoderma spp.	Metal-cyanide	2000mg.L^{-1}	6.5	25
Cryptococcus humicolus	Tetra-cyanide-nickelate (II)	51mM	7.5	25

Source: Amended from DASH; GAUR; BALOMAJUMDER, 2009

Cyanide-degrading microorganisms has been previously isolated from natural waters (LUQUE-ALMAGRO et al., 2005a), gold mine tailings (RAZANAMAHANDRY et al., 2016; MONGE et al., 2017), metal-plating effluents (WATANABE et al., 1998; MEKUTO et al., 2013), soil (ADJEI; OHTA, 1999; NWOKORO; DIBUA, 2014), cassava-processing wastewater (KANDASAMY et al., 2015), sago industry effluents (SANKARANARAYANAN; GOWTHAMI, 2015), coke oven wastewater (MIRIZADEH; YAGHMAEI, 2014) and jewelry effluents (SABATINI et al., 2012). These studies have shown that microbes adapt to hostile environments and therefore, cyanide-degrading species can be isolated from cyanide-containing wastes and they could be adapted to toxic concentrations as high as the one present in the waste from which they were adapted.

Cyanide can be used as a nitrogen or carbon source for microorganisms, or it can be used in non-synthesis metabolic pathways that reduce cyanide toxicity. The effectiveness of using a specific strain thus depends on its ability to degrade cyanide under certain conditions. These conditions are usually similar to the characteristics of the sample from where the bacteria were isolated since the organisms are already adapted to that environment. In that sense**,** temperature, initial cyanide concentration, pH, and aeration are external factors that contribute to biodegradation of cyanide (GURBUZ; CIFTCI; AKCIL, 2009). Therefore, culture-based methods have assessed different nutrient sources for cyanide-degrading bacteria. Table 5 presents carbon and nitrogen sources in previously reported studies for bacterial cyanide degradation as well as pH and temperature conditions that are essential to promote cellular growth.

Table 5. Conditions used for cyanide degradation by different organisms

Microorganism	**C-source**	**N-source**	**Temp °C**	**pH**
Pseudomonas sp.	Whey	WAD CN	30	9.2-11.4
Fusarium solani	Glucose	$K_2Ni(CN)_4$	25	7
Pseudomonas fluorescens	Glucose	$Fe(CN)_6$	25	5
Rhodococcus sp.	KCN	KCN	30	-
Klebsiella oxytoca	Glucose	CN, SCN	30	7
Trichoderma ssp.	Glucose	CN^-	25	6.5
Trametes versicolor	Citrate	KCN	30	10.5
Scenedesmus obliquus	NaCN	NaCN	-	10.3

Source: Adapted from (MEKUTO; NTWAMPE; AKCIL, 2016)

Considering the conditions in which microorganisms are tested, cyanide degradation can present rates as low as 20% or as high as 90% from initial cyanide concentration. Sujata et al. achieved 64% degradation of free cyanide using a native *Bacillus pumilus* strain and glucose as a carbon source (KANDASAMY et al., 2015). Nallapan et al. achieved complete cyanide degradation of 0.1mM KCN within 24 hours incubation using glucose and yeast extract as nutrients for the growth of a *Rhodococcus* UKMP-5M strain (NALLAPAN MANIYAM et al., 2013). Huertas et al. achieved 60% of cyanide degradation in alkaline condition by *Pseudomonas pseudoalcaligenes* CECT5344 in a batch reactor (HUERTAS et al., 2010). In general, individual strains present different degradation rates when incubated at different conditions.

For example, Karamba et al. tested several incubation conditions for a cyanide-degrading *Serratia marcescens* strain and determined that cyanide attenuation was optimal at pH 6, temperature 32.5˚C, inoculum size 20% and cyanide initial concentration of 200mg.L^{-1} (KARAMBA et al., 2018).

Authors have also tested cyanide degradation by consortia (MEKUTO et al., 2013; SANKARANARAYANAN; GOWTHAMI, 2015). Using more than one stain at a time, cyanide degradation occurs by several enzymatic pathways simultaneously, or symbiotic associations can be developed between different strains which ensure quality and sustainability for remediation processes (RAZANAMAHANDRY et al., 2016).

3.5.3.3 CYANIDE-CONTAINING EFFLUENTS BIOTREATMENT

BIotreatment of cyanide-containing effluent has been widely studied in the mining industry (ATA AKCIL, 2003; GURBUZ; CIFTCI; AKCIL, 2009; KUYUCAK; AKCIL, 2013; LUQUE-ALMAGRO; MORENO-VIVIÁN; ROLDÁN, 2016; RAZANAMAHANDRY et al., 2016). For mine operations, biotreatment of cyanide effluents represents a low cost in operation and installation to treat several pollutants at the same time (BOTZ; MUDDER; AKCIL, 2016). *Pseudomonas sp* is the most widely used strain for cyanide treatment and several authors have proven the efficiency of this species in cyanide treating; however, its application is still reduced to a few conditions (WATANABE et al., 1998; HUERTAS et al., 2010; NWOKORO; DIBUA, 2014; LUQUE-ALMAGRO; MORENO-VIVIÁN; ROLDÁN, 2016; SINGH; BALOMAJUMDER, 2016).

Commercial application of cyanide biodegradation uses active, passive and *in situ* technologies (AKCIL et al., 2003). Active systems have been installed full-scale to treat cyanide-containing effluents. Although, only when cyanide concentrations and metal concentrations are non-toxic for conventional biological systems (AKCIL, 2003). Meanwhile, passive systems such as wetlands, have been installed to treat drainage containing cyanide collected from heap leach pad, or pit lakes (KUYUCAK; AKCIL, 2013).

Biodegradation has been studied for applicability in the treatment of leaching wastes (DÍAZ; CAIZAGUANO, 1999). In industrial scale, cyanide biotreatment has been applied in the Homestake Mining Company in South

Dakota, USA. This mining site used 48 Rotating Biological Contactors (RBC) in a patented process to attach biofilm where an isolated strain of *Pseudomonas paucimobilis* with an inorganic carbon source grows, achieving 99% removal of total cyanide and 95-98% removal of copper (WHITLOCK, 1990). The same company later developed new technologies for cyanide treatment including one based on a suspended sludge system followed by a denitrification process, and one Biopass process designed to treat drain down of a heap leach operation (BAXTER; CUMMINGS, 2006).

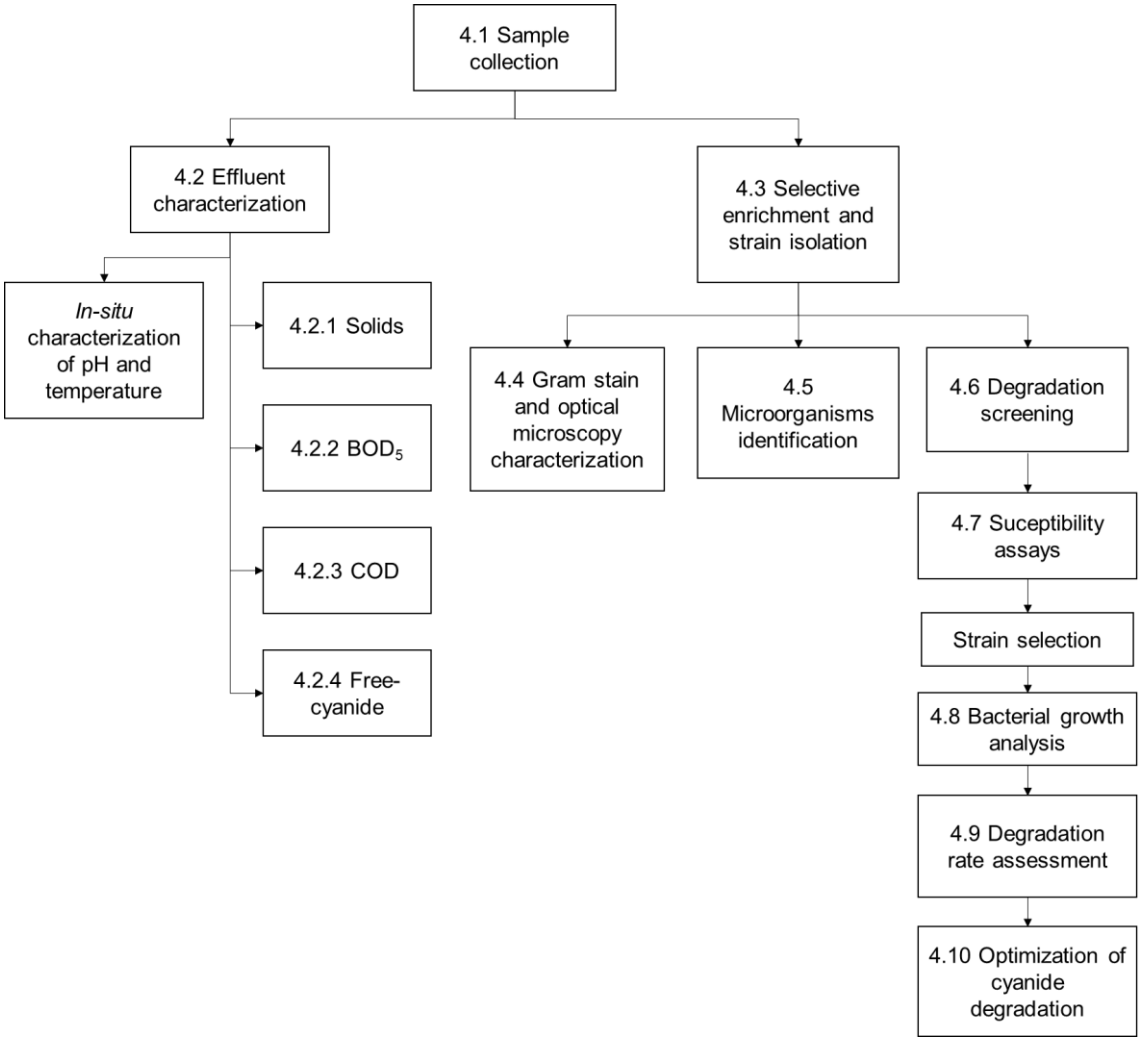
Other industrial applications of cyanide biotreatment have tested the addition of nutrients for bioprospection of native organisms. The US Bureau of Mines studied the viability of bacterial cyanide oxidation at USMX-s Green Springs mine using a *Pseudomonas pseudoalcaligenes* isolated strain in tailing pond containing 280mg.L^{-1} CN^- achieving a concentration of 8.5mg.L^{-1} after a 15-week incubation period (LIEN; ALTRINGER, 1993). Moreover, Pintail Systems Inc. conducted a full-scale cyanide detoxification project at a heap leach pad in the Yellow Pine Mine (Idaho, USA) enhancing native bacteria by adding nutrients, although the system was later affected by cold temperature operations (USEPA, 1994).

Industrial-scale projects on cyanide biodegradation show the potential of this technology. Bacterial treatment does not generate any new waste flow that could represent an environmental risk. Furthermore, it does not involve hazardous chemical handling. However, the technology still needs to be studied and developed to achieve more efficient results. In order to assess the applicability of biological treatment, a two-phase program has to be employed involving first the isolation of microorganisms and a bench-scale laboratory study followed by a continuous flow pilot plant evaluation (AKCIL, 2003). Furthermore, microbial communities involved in the degradation of cyanide have to be understood since operational parameters such as temperature, pH, cyanide concentration, and aeration are crucial in the operational research for cyanide degradation (MEKUTO; NTWAMPE; AKCIL, 2016).

4 MATERIALS AND METHODOLOGY

This study isolated cyanide-degrading microorganisms from a cassava-processing effluent and assessed their potential for free-cyanide treatment. Figure 8 illustrates the methodology used to characterize the cassava-processing effluent used in this study, the isolation of cyanide-degrading bacteria and the procedure to optimize cyanide degradation by selected isolated strains. A more detailed explanation of each step is described below.

Figure 8. Methodology for cassava-processing effluent characterization and cyanide-degrading bacteria isolation and characterization



Source: Personal collection

4.1 SAMPLE COLLECTION

A cassava-processing industry is located in Santa Maria da Serra, in the state of São Paulo, Brazil. The industry produces approximately 35tonnes of

cassava flour per day. All the production occurs from cassava roots, the process uses a wide variety of cassava varieties, all of them belonging to the bitter types of cassava (locally known as "*mandioca brava*"). The industry only produces traditional cassava flour by a semi-automatic method. The system consists of a reception unit, washing and peeling process, a grating unit, dewatering equipment, a drying unit, and a packaging area.

Effluents from the washing and the dewatering processes are kept separately for treatment and disposition. Effluent samples were collected from the dewatering process. The sample collection procedure was performed according to the Standard Method for the Examination of Water and Wastewater (APHA/AWWA/WEF, 2012). A total of 3L of sample were collected: 1L was collected in a sterile glass bottle for microorganism isolation, 1L in a plastic bottle without preservation for solids, BOD_5 and COD determination. 1L in a plastic bottle preserved with concentrated NaOH until pH12 for free-cyanide characterization.

Collected samples were transported in cooler kept at 4°C to the Laboratory of Recycling, Waste Treatment and Extraction (LAREX) of the Escola Politécnica - Universidade de São Paulo for further analyses.

4.2 EFFLUENT CHARACTERIZATION

Because of the instability of certain parameters, they are measured *in-situ* and analyzed as possible after sample collection (APHA/AWWA/WEF, 2012). Temperature and pH can change rapidly and the value of these parameters can affect the concentration of organic compounds. These parameters were thus measured *in-situ*.

In order to have a general vision of the effluent to be treated, it is important to know the processes that originated the waste as well as the characterization of chemical parameters that influence its biodegradation potential including organic matter content, solids content and toxic substances concentration (LIMA et al., 2007). Hence, further *ex-situ* characterization was performed by analyzing solids, Biochemical Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD) and free-cyanide in an effluent sample.

4.2.1 SOLIDS

The presence of solids in water begins from the dissolution of salts and the presence of insoluble substances in water. Solids affect water and wastewater quality and is a key parameter in the control of biological and physical wastewater treatment processes (APHA/AWWA/WEF, 2012)

Total Solids (TS), Suspended Solids (SS) and Dissolved Solids (DS) were measured according to 2540 Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF, 2012).

For Total Solid (TS) porcelain dishes were prepared by cleaning with ultrapure water and drying to 104°C for 2h. Then, dishes were cooled to room temperature in desiccator and weight before use. A 50mL volume aliquot of homogenous sample was put in each porcelain dish. Samples were dried in a stove at 104°C until constant weight, cooled to room temperature in a desiccator, and weighed. Effluent sample was analyzed in triplicates. Total Solids (TS) were calculated according to Equation 10.

$$TS\ (mg.L^{-1}) = \frac{w_f - w_d \times 1000}{Sample\ volume\ (mL)} \qquad \text{Equation 10}$$

Where w_f is the weight of the dried dish containing the residue and w_d is the weight of the dried dish before adding sample, both in mg.

In order to measure Suspended Solids (SS), 3µm pore glass fiber filters were prepared by inserting them in a vacuum filtration apparatus. Then, filters were washed using 20mL of ultra-pure water 3 successive times. Washings were discarded and filters were transferred to aluminum weighing dishes and drought in a stove at 104°C for 2h. Filters were cooled in a desiccator and weighed before use. Filtration apparatus was assembled and vacuum was started. A 100mL volume aliquot of well-mixed sample was filtered and three successive washings using 20mL of ultra-pure water were done to make sure no sample was left in the funnel. Filters were carefully removed, transferred to an aluminum dish, dry in a stove at 104°C until constant weight, cooled to room temperature in a desiccator and weighed. Sample was analyzed in triplicates and the result reported is the

average between them. Suspended Solids (SS) was calculated according to Equation 11.

$$SS\ (mg.L^{-1}) = \frac{w_f - w_d \times 1000}{Sample\ volume\ (mL)} \qquad \text{Equation 11}$$

Where w_f is the weight of the dried filter containing the residue and w_d is the initial weight of the dried filter, both in mg.

According to the 2540 Method (APHA/AWWA/WEF, 2012), the difference between the Total Solids (TS) and the Suspended Solids (SS) in the sample, corresponds to the concentration of Dissolved Solids (DS), since solids in an aqueous solution can only be either suspended or dissolved.

4.2.2 BIOCHEMICAL OXYGEN DEMAND (BOD_5)

Microorganisms grow in wastewaters consuming organic matter. These microorganisms can use some pollutants as nutrients source, including toxic compounds as cyanide. In the biotreatment of industrial effluents, it is important to measure the Biochemical Oxygen Demand as an indicator of the biodegradable organic matter present in the effluent. Therefore, BOD_5 was measured according to 5210 Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF, 2012).

Table 6. Biochemical Oxygen Demand test solutions

Solution	Name	Composition
A	Phosphate buffer Solution	8.5g.L^{-1} KH_2PO4, 21.75 g.L^{-1} K_2HPO_4, 33.4g.L^{-1} $Na_2HPO_4.7H_2O$ and 1.7g.L^{-1} NH_4Cl pH should be 7.2
B	Magnesium sulfate solution	22.5g.L^{-1} $MgSO_4.7H_2O$
C	Calcium chloride solution	27.5g.L^{-1} $CaCl_2$
D	Ferric chloride solution	0.25g.L^{-1} $FeCl_3.6H_2O$

Source: Personal Collection

First, solutions A, B, C, and D were prepared with ultra-pure water according to Table 6. Dilution water was prepared in enough volume in a clean volumetric flask adding ultra-pure sterile aerated water, for dissolved oxygen

(DO) concentration approach saturation, and adding 1mL of each A, B, C, and D solutions. Dilution water was prepared and used immediately.

Effluent sample was collected and kept at 4°C until characterization. BOD_5 measurement was performed 24hours after sample collection to assure quality in the analysis. Five dilutions and a blank were prepared using dilution water in a proportion estimated to produce residual DO of at least 1mg.L^{-1} and a DO consumption of at least 2mg.L^{-1} after a 5-days incubation. For the cassava-processing effluent, and according to previously-executed effluent monitoring at the industry site, dilutions containing 0.3, 1, 1.7 and 2.5% (v/v) were prepared in 300mL glass bottles with ground-glass stoppers. Bottles were washed and rinsed with ultra-pure water and drought before use.

Each bottle was filled with dilution water and initial DO was measured using a DO probe with a stirrer; then, bottles were tapped making sure there are no air bubbles in the bottle. Bottles were incubated at 20°C for five days. After the incubation period, DO was measured in each bottle and BOD_5 was calculated according to Equation 12.

$$BOD_5\ mg.L^{-1} = \frac{DO_f - DO_i}{P} \qquad \text{Equation 12}$$

Where DO_f is the final concentration of dissolved oxygen in the bottle, DO_i is the initial dissolved oxygen concentration in the bottle and P is the volumetric fraction of sample used (volume of sample in mL/volume of bottle in mL).

4.2.3 CHEMICAL OXYGEN DEMAND (COD)

The Chemical Oxygen Demand (COD) is the amount of oxygen needed to oxidize all the inorganic and organic matter present in a sample. COD was measured according to the method DIN ISO15705 and it is analogous to the United States Environmental Protection Agency (USEPA) Method 410.4: Determination of Chemical Oxygen Demand by Semi-Automated Colorimetry (USEPA, 1993).

The measuring range was between 100 and 1500mg.L^{-1} COD. Calibration curve was prepared using standard solutions containing 0, 100, 250, 500, 1000, and 1500mg.L^{-1} of COD from a potassium hydrogen phthalate ($C_8H_5O_4K$)

1000mg.L^{-1} stock solution. COD of potassium hydrogen phthalate was calculated stoichiometrically according to Equation 13 to be 1.17mgCOD/mg$C_8H_5O_4K$.

$$C_8H_5O_4K + \frac{15}{2}O_2 \rightarrow 8CO_2 + 2H_2O + K^+ + OH^- \quad \text{Equation 13}$$

For sample preparation, glass tubes were cleaned and washed from any impurities. Then, it was added 0.3mL of Solution A (Cat No. 114538, Merck) and 2.3mL of Solution B (Cat. No. 114539, Merck) to each tube. Tubes were closed and any sediment was suspended by swirling. Carefully, 3mL of the sample (diluted 1:100) or standard solution was added to each tube and heated at 148ºC for 120minutes in a thermoreactor. Tubes were swirled and left to cool to room temperature and measure in a photometer at wavelength 605nm.

4.2.4 FREE-CYANIDE

Free-cyanide concentration, including HCN and CN- forms of cyanide, was measured using a polarographic method, by a standard-addition determination. The data were obtained by using a voltammetric analyzer (VA) equipment Computrace Control 797 model, Metrohm. The polarographic method used in this study is not interfered by the presence of sulfides in solution (CANTERFORD, 1975). Samples were diluted using a KOH 0.01M solution in order to have a free-cyanide concentration between 0.01 and 10mg.L^{-1}. Supporting electrolyte solution (0.2M H_3BO_3 and 0.17M KOH) and 0.01M KOH solutions were prepared according to the manufacturer (METROHM,).

4.3 SELECTIVE ENRICHMENT AND BACTERIA ISOLATION

A selective medium for cyanide-resisting bacteria selection was prepared. This medium was modified from (WU et al., 2014) and contained NH_4Cl 1g.L^{-1}, KH_2PO_4 0.5g.L^{-1}, K_2HPO_4 1.5g.L^{-1}, $MgSO_4$ 0.2g.L^{-1}, NaCl 0.5g.L^{-1}, starch 0.5g.L^{-1} and sterilized by autoclaving at 121ºC for 30 minutes and was supplemented in 1% (v/v) with a cyanide-concentrated solution (37.7mgNaCN.mL^{-1} 75% purity in 0.25%(w/v) NaOH), sterilized by filtration using a 0.22µm pore filter, to obtain a final pH of 8 and a final concentration of 150mg.L^{-1} of free-cyanide. Solid selective media was prepared by adding 18g.L^{-1} of agar sterilized separately by autoclaving at 121ºC for 30 minutes.

An aliquot of 100µL of the cassava-processing effluent was inoculated in a selective medium plate, incubated at 30°C, and checked daily for colony formation for five days. Then, mixed cultures with different morphology were isolated and inoculated in a new plate containing LB medium (15g.L^{-1} peptone, 5 g.L^{-1} yeast, 10 g.L^{-1} NaCl, and 15 g.L^{-1} agar) by streaking technique until single species were isolated.

4.4 GRAM STAIN AND OPTICAL MICROSCOPY

Isolated strains were characterized by the Gram stain technique, which is based on the ability of the cells in retaining the crystal violet dye due to the presence of certain structures in the cell wall of the microorganism.

Gram stain and optical microscopy characterization is the first step to identify a microbial strain. The difference between Gram-positive and Gram-negative bacteria is the composition of the cellular wall. Gram-positive organisms possess a thick cell wall compared to Gram-negative bacteria that have a thinner cellular wall but, at the same time, possess an additional outer membrane, which gives cells different characteristics when responding to stress, heat, or antibiotics (MAI-PROCHNOW et al., 2016)

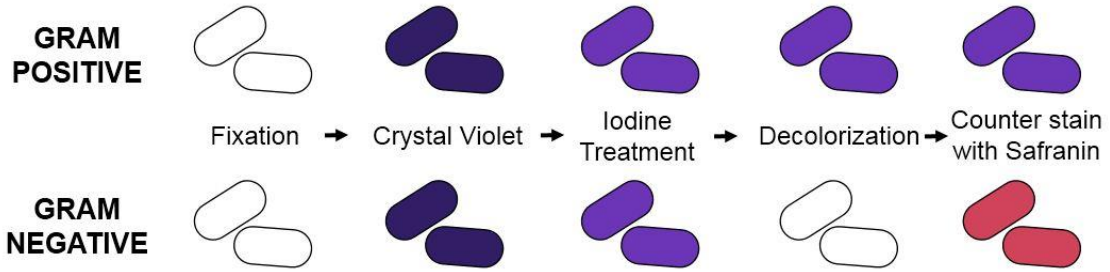
In order to evaluate the morphological characteristics of the isolated microorganisms, each strain was Gram-stained and analyzed by optical microscopy (Leica DM2700 M). The Gram stain technique requires the preparation of four solutions: a crystal violet solution, an iodine solution, a decolorizer solution, and a safranin solution. For Gram Crystal Violet Solution, 1mL of crystal violet stock solution (20g of crystal violet dissolved in 100mL of ethanol) was mixed with 40mL of oxalate stock solution (1g of ammonium oxalate in 100mL of water) and 10mL of water. For Gram Iodine Solution, 1g of iodine, 2g of potassium iodide and 3g of sodium bicarbonate were dissolved in 300mL of water. For Decolorizer Solution, equal volumes of 95% ethanol and acetone were mixed. Finally, for Gram Safranin Solution, 1mL of safranin stock solution (2.5g of safranin in 100mL of 95% ethanol) was mixed with 5mL of water.

Each strain was cultivated in LB medium plates (15g.L^{-1} peptone, 5 g.L^{-1} yeast, 10 g.L^{-1} NaCl, and 15 g.L^{-1} agar) and incubated in a stove at 30°C overnight. A 20µL of sterile ultra-pure water was added on a clean microscope

slide and a colony was transferred from the plate using an inoculation loop, spread and left to air-dry in a sterile environment.

The Gram stain technique consists of five steps illustrated in Figure 9. First, two drops of Gram crystal violet solution were added to the fixed culture. After 1min, the slide was gently rinsed using ultra-pure water. Then, two drops of Gram iodine solution, or enough to cover the culture, was poured. After 1min, the slide was rinsed with ultra-pure water carefully to not wash off the culture from the slide. Then, a few drops of the decolorizer solution were added and the slide was rinsed with ultra-pure water after five seconds. Finally, culture was counterstained with three drops of safranin solution, let stand for 30 seconds, and washed off the solution using ultra-pure water. The microscope slide was let to air-dry and then analyzed by Leica DM2700 M optical microscope using a 100X oil immersion objective lens.

Figure 9. Gram stain technique



Source: Personal collection

4.5 MICROORGANISMS IDENTIFICATION

Isolated bacteria were identified by Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry technique using a Microflex LT model equipment (Bruker Daltonics).

Isolated strains were individually grown in LB plates (15g.L^{-1} peptone, 10g.L^{-1} NaCl, 5g.L^{-1} yeast, and 15g.L^{-1} agar) and incubated in a stove at 30°C overnight. Strains were cultivated by streaking four times on the same plate using a sterile stick to assure the cultivation of individual strains.

Grown strains were transferred to the analysis plate (Ground Steel MALDI Target Plates, Bruker Daltonics) using a sterile stick and 1µL of the matrix solution was added. Matrix solution was prepared according to the manufacturer using α-cyano-4-hydroxycinnamic acid saturated in a solvent solution containing 30:70 (v/v) acetonitrile : 0.1% trifluoracetic acid in water (BRUKER DALTONICS, 2015a). The plate was left to dry in a sterile environment and analyzed to produce protein mass spectrometry spectra of each strain.

Bacteria identification was performed by matching obtained spectra with protein patterns, specific for each species, using Biotyper software (Version 2.0, Bruker Daltonics). The spectrum of each organism is analyzed by the software using a biostatistical algorithm and comparing it to a list of organisms in the reference library, score that similarity between 0 and 3 and categorized it according to Table 7 (BRUKER DALTONICS, 2015b).

Table 7. Meaning of score values for bacterial identification using Biotyper software

Range	Interpretation	Color
2.300 – 3.000	Highly probable species identification	Green
2.000 – 2.299	Secure genus identification, probable species identification	Green
1.700 – 1.999	Probable genus identification	Yellow
0.000 – 1.699	Not reliable identification	Red

Source: (BRUKER DALTONICS, 2015b)

4.6 DEGRADATION SCREENING

After isolated strains were obtained from the cassava-processing effluent, they were tested individually for their ability to degrade free-cyanide. In order to achieve this, each strain was cultivated in LB solid medium plates (15g.L^{-1} peptone, 10g.L^{-1} NaCl, 5g.L^{-1} yeast, and 15g.L^{-1} agar) and incubated in a stove at 30°C overnight. Then, a colony of each strain was transferred to a 50mL falcon tube containing 15mL of LB liquid medium (15g.L^{-1} peptone, 10g.L^{-1} NaCl, and 5g.L^{-1} yeast) and incubated at 30°C 185RPM until Optical Density at λ=600nm (OD600) was 1. Each tube was then centrifuged at 8000RPM for five minutes and washed twice using a saline solution (NaCl 0.9%). Then, tubes were

centrifuged once more and pellet was re-suspended in solution containing NH_4Cl 1g.L^{-1}, KH_2PO_4 0.5g.L^{-1}, K_2HPO_4 1.5g.L^{-1}, $MgSO_4$ 0.2g.L^{-1}, NaCl 0.5g.L^{-1}, CH_3COONa 1g.L^{-1}, Tris buffer 3g.L^{-1}, supplemented with NaCN to a final concentration of 150mg.L^{-1} CN^-, regulated to pH 8 and sterilized by filtration. Tubes were incubated in orbital agitation at 30°C 185RPM for 72h. A negative control containing no bacteria was carried out under the same incubation conditions. After the incubation period, residual cyanide was analyzed to calculate the degradation percentage using Equation 14. All assays were executed in triplicates and results were presented as the average between triplicates. The strains with more degradation potential were selected for further assays.

$$\%[CN]d = \frac{[CN]_i - [CN]_f}{[CN]_i} \times 100\% \qquad \text{Equation 14}$$

Where %[CN] is the percentage of free-cyanide degraded by strain or in the blank; $[CN]_i$ is the initial concentration of free-cyanide in solution (mg.L^{-1}); and $[CN]_f$ is the final concentration of free-cyanide in solution after the incubation period (mg.L^{-1}).

4.7 SUSCEPTIBILITY ASSAYS

Selected strains were tested for their ability to degrade cyanide under stress conditions. This analysis was performed by growing individual strains in 50mL falcon tubes containing 15mL of LB medium (15g.L^{-1} peptone, 10g.L^{-1} NaCl, and 5g.L^{-1} yeast) and incubated at 30°C 185RPM overnight. Optical density of each tube was adjusted to 1. Tubes were centrifuged at 8000RPM for 5min and washed twice using a saline solution (NaCl 0.9%). Then tubes were centrifuged, the supernatant was discarded and pellet was re-suspended in assay solution and incubated at 30°C 185RPM for 48h.

To assess susceptibility of strains for cyanide degradation to initial free-cyanide concentration, assay solution contained NH_4Cl 1g.L^{-1}, KH_2PO_4 0.5g.L^{-1}, K_2HPO_4 1.5g.L^{-1}, $MgSO_4$ 0.2g.L^{-1}, NaCl 0.5g.L^{-1}, CH_3COONa 1g.L^{-1}, Tris buffer 3g.L^{-1}, supplemented with NaCN to a final concentrations of 150, 300, 500 and 1000mg.L^{-1} CN^-, regulated to pH 8 and sterilized by filtration with a 0.22µm diameter pore filter.

To assess susceptibility to pH of cyanide degradation by the isolated strains, values of 8, 9, 10, and 11 were tested. In this case, assay solution contained NH_4Cl 1g.L^{-1}, KH_2PO_4 0.5g.L^{-1}, K_2HPO_4 1.5g.L^{-1}, $MgSO_4$ 0.2g.L^{-1}, NaCl 0.5g.L^{-1}, CH_3COONa 1g.L^{-1}, supplemented with NaCN to a final concentration of 150mg.L^{-1} CN^- and Tris buffer 3g.L^{-1} for pH values of 8 and 9, and CAPS 5.53g.L^{-1} for pH values of 10 and 11. pH was regulated using a concentrated HCl solution or a NaOH 5M solution and the assay solution was sterilized by filtration with a 0.22µm diameter pore filter.

Residual cyanide was measured after the incubation period as described in section 4.2.4 and cyanide degradation was calculated according to Equation 14. All assays were executed in triplicates and results were presented as the average between triplicates. Most resistant strains were selected for further assays.

4.8 BACTERIAL GROWTH ANALYSIS

In order to achieve the highest cyanide degradation capacity, microorganisms should be in their exponential phase of growth, so that they are producing every enzyme they need for their metabolism. In order to harvest cyanide-degrading microorganisms in their exponential growth phase, growth curves were constructed for each species by inoculating 1% (v/v) of selected strains in fresh sterile LB medium (15g.L^{-1} peptone, 10g.L^{-1} NaCl, and 5g.L^{-1} yeast) and incubating flasks at 30°C 185RPM. Aliquots were taken every 1.5h to assess microbial growth. Microorganism concentration was measured simultaneously by optical density at λ=600 and by using the Miles and Misra method to quantify colony forming units (MILES; MISRA; IRWIN, 1938). Growth curves were constructed for each strain until the stationary phase of growth was achieved.

4.9 DEGRADATION RATES ASSESSMENT

The kinetics and rates of cyanide degradation by each strain were studied by performing degradation assays during 24h. Individual strains were grown in LB medium (15g.L^{-1} peptone, 10g.L^{-1} NaCl, and 5g.L^{-1} yeast) until exponential growth is achieved based on the results of the previous section. Cells were harvested by centrifugation at 8000RPM for 5min and washed twice using a

saline solution (NaCl 0.9%). Then tubes were centrifuged and pellet was resuspended in degradation-assay solution and incubated at 30°C 185RPM. Degradation solution contained NH_4Cl 1g.L^{-1}, KH_2PO_4 0.5g.L^{-1}, K_2HPO_4 1.5g.L^{-1}, $MgSO_4$ 0.2g.L^{-1}, NaCl 0.5g.L^{-1}, CH_3COONa 1g.L^{-1}, Tris buffer 3g.L^{-1} supplemented with NaCN to a final concentration of 150mg.L^{-1} CN^- and pH regulated to 8. An aliquot was taken each 4hours to measure residual cyanide, bacterial concentration by optical density and colony formation units, and pH.

4.10 OPTIMIZATION OF CYANIDE DEGRADATION

Understanding the conditions influencing the biological degradation of cyanide may yield important information leading to the optimization of its use in cyanide treatment. In that order, the present study assessed the influence of using different carbon sources, temperature and rotation speed conditions during the incubation in the biological degradation of cyanide by each selected individual strain.

4.10.1 INFLUENCE OF CARBON SOURCE

Cyanide degrading microorganisms are able to assimilate cyanide as a nitrogen source, but not as a carbon source (MIRIZADEH; YAGHMAEI, 2014). Therefore, cyanide treatment processes need a supply of a carbon source. This study used starch, sodium acetate, and glycerol as carbon sources.

To assess the influence of the carbon source in cyanide degradation, microorganisms were harvested after being cultures in LB médium incubated at 185RPM 30°C until optical density equal to 0.8. Culture was centrifuged at 8000RPM for 5min and washed twice using a NaCl 0.9% solution. Pellet was resuspended in assay solution containing NH_4Cl 1g.L^{-1}, KH_2PO_4 0.5g.L^{-1}, K_2HPO_4 1.5g.L^{-1}, $MgSO_4$ 0.2g.L^{-1}, NaCl 0.5g.L^{-1}, Tris buffer 3g.L^{-1} supplemented with NaCN to a final concentration of 150mg.L^{-1} CN^- and pH regulated to 8 and 1g.L^{-1} of sodium acetate, starch or glycerol as a carbon source. Flasks were incubated at 30°C 185RPM for 24h and residual cyanide was measured according to the procedure in section 4.2.4.

4.10.2 INFLUENCE OF INCUBATION CONDITIONS

Degradation assays using individual isolated and selected strains were performed in orbital agitation in fixed temperature and rotation speed conditions. In order to achieve this, 15mL of microbial culture in the exponential phase of growth (OD=0.8) was harvested by centrifugation at 8000RPM for 5min and washed twice using a sterile saline solution (NaCl 0.9%). Then, pellet was resuspended in 15mL of assay solution containing NH_4Cl 1g.L^{-1}, KH_2PO_4 0.5g.L^{-1}, K_2HPO_4 1.5g.L^{-1}, $MgSO_4$ 0.2g.L^{-1}, NaCl 0.5g.L^{-1}, CH_3COONa 1g.L^{-1}, Tris buffer 3g.L^{-1} supplemented with NaCN to a final concentration of 150mg.L^{-1} CN^- and pH regulated to 8. Degradation rates were calculated by measuring residual cyanide following steps in section 4.2.4 and microbial concentration by colony formation units by the Miles and Misra method (MILES; MISRA; IRWIN, 1938) every 6h for 24h. In this study, the influence of the temperature and speed rotation condition in cyanide degradation was assessed by testing one factor at a time. Temperatures tested were 30, 25 and 20°C in order to assess if the process could still occur in environmental temperatures. Speed rotation was tested using 135, 185 and 235RPM values.

5 RESULTS AND DISCUSSION

5.1 SAMPLE COLLECTION AND EFFLUENT CHARACTERIZATION

A sample of cassava-processing effluent was collected from a cassava flour industry located in Santa Maria da Serra, São Paulo, Brazil. The results of the physical-chemical characterization of the sample are shown in Table 8.

Table 8. Physical and chemical characteristics of effluent collected from a dewatering process in cassava flour production

Parameters	**Findings**
Temperature	19 °C
pH	5.5
BOD_5	45689 $mgO_2.L^{-1}$
COD	71254 $mgO_2.L^{-1}$
Total Solids	63.32 $g.L^{-1}$
Suspended Solids	6.7 $g.L^{-1}$
Dissolved Solids	56.6 $g.L^{-1}$
Free-cyanide	305 $mg.L^{-1}$

Temperature and pH were measured *in situ* to assure the less variability possible. At the cassava flour factory, effluent is discarded at an environmental temperature which is between 10 – 29 °C in Santa Maria da Serra region depending on the season. Registered temperature is originated by the water temperature used in the grating process as there is no heating or cold during the process.

The effluent presented a pH value of 5.5. At this value, the transformation of cyanogenic glycosides from acetone cyanohydrin to acetone and free cyanide occurs spontaneously. The liberation of free-cyanide from cassava pulp is thus limited only by linamarase activity, the enzyme that catalyzes the decomposition of linamarin (MCMAHON; WHITE; SAY, 1995). However, this pH value is also below the pKa value of cyanide (9.2) meaning that free cyanide present in solution is mainly in the form of HCN and not CN^- promoting the volatilization of cyanide.

Indirect determination of the biodegradable organic matter and total organic matter in the effluent was assessed by measuring BOD_5 and COD in the sample, respectively. The 5-day Biochemical Oxygen Demand (BOD_5) is an indirect method to assess the concentration of biodegradable organic matter in a liquid sample. The BOD_5 is the amount of oxygen needed to stabilize the biodegradable organic matter in a sample of water by the microorganism naturally present in it (LIMA et al., 2007). The cassava-processing effluent presented a BOD_5 of 45689 $mgO_2.L^{-1}$ using only the native microorganisms present in the effluent, without the addition of seeds. The consumption of oxygen by native microorganisms in the sample shows that the sample has a microbiota adapted to effluent that is able to oxidize organic matter in those conditions. The cassava-processing effluent presents a BOD_5 125 times higher than an average domestic sewage (LIMA et al., 2007) which indicates its potential risk for polluting natural waters and its need to be treated prior to discharge to the environment.

The COD indicates the presence of total organic and inorganic matter subject to oxidize in sample, including biodegradable and non-biodegradable organic matter. Whereas BOD_5 measures the oxygen consumption by native organisms to oxidize biodegradable organic matter, COD uses a strong oxidizing agent, usually potassium dichromate, to fully oxidize all components subject to oxidation in the sample. COD is expressed in $mg.L^{-1}$ COD which is equal to $mgO_2.L^{-1}$. Average cassava-processing wastewaters present COD values between 600 and 50000$mgO_2.L^{-1}$ (KAEWKANNETRA et al., 2009; KHONGKLIANG et al., 2017), the effluent sample presented a COD of 71254$mgO_2.L^{-1}$. This value is a reference for the high content of organic matter in the effluent which can be dangerous when discharged carelessly to the environment.

The COD:BOD_5 ratio was calculated in Equation 15. Effluents with this ratio lower than 2 are considered to be easily biodegradable (LIMA et al., 2007) which indicates that they have native microbiota capable of consuming organic matter in the effluent. Other cassava-processing wastewaters can present a higher ratio when the inhibitory action of cyanide affects the biodegradation processes (COLIN et al., 2007). Nonetheless, cassava-processing effluents have

nutrients in enough concentrations to promote bacterial growth when microorganisms are adapted to the presence of toxic substances.

$$\frac{COD}{BOD} = \frac{71254\text{mg}O_2.\text{L}^{-1}}{45689\text{mg}O_2.\text{L}^{-1}} = 1.55 \qquad \text{Equation 15}$$

The cassava-processing effluent presented 63.32g.L^{-1} of total solids. Dissolved solids represent the biggest fraction of solids (89%). Because the waste comes from the dewatering process of cassava flour production, it is to expect that most suspended solids are separated in the press that generates the effluent. However, other cassava-processing industries produce effluents with a lower presence of solids, generally between 4.5 and 38.2g.L^{-1} of total solids (ZHANG et al., 2016).

The concentration of free-cyanide in the effluent was 305mg.L^{-1} which indicates that free-cyanide is constantly formed in the effluent due to the presence of cyanogenic glycosides, the pH and the temperature of the sample; and that it is maintained despite the pH value being lower than the pKa of cyanide. This occurs probably because cyanide is constantly being liberated while it is volatilizing at the same time. The cyanide content in cassava-processing effluents can vary depending on the type of process or the operation that generates the waste. In general, authors explain that starch production wastewaters present a lower concentration of cyanide, typically below 10mg.L^{-1} while flour production wastewaters typically present cyanide concentrations from 10 to 20mg.L^{-1} but can also reach 100mg.L^{-1} (DE CARVALHO et al., 2018). The concentration of cyanide in the sample used in this study is higher than the average reported by authors, this is probably influenced by the variety of cassava used in the process and by the time between the production of the wastewater, the collection of the sample, and the determination of cyanide in the laboratory.

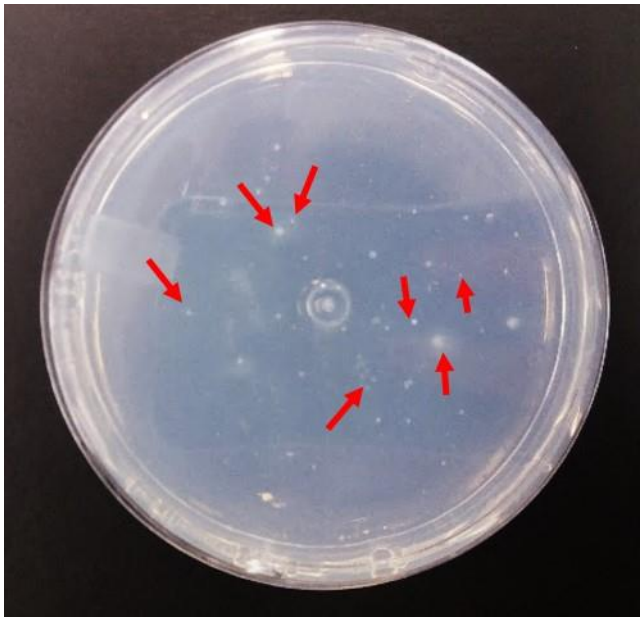
As the organic matter content in the sample is high, the temperature and the pH are within physiological values and the COD:BOD_5 ratio indicates that the effluent would be easily biodegradable, it is expected to find microbiota adapted to effluent conditions in the sample, including the presence of toxic compounds such as cyanide.

5.2 SELECTIVE ENRICHMENT AND STRAIN ISOLATION

The selection of microorganisms from the autochthonous microbiota was performed using a selective enrichment technique. Microorganisms that survived the presence of cyanide were cultivated in plates with minimum medium enriched with cyanide and starch as sources of nitrogen and carbon, respectively.

Figure 10 shows the inoculated plate after 5 days of incubation at 30˚C and the 6 points from where mix cultures were taken for streaking isolation. Individual colonies with different morphology were isolated by streaking four times in new plates containing LB solid medium. After streaking, a total of 16 individual strains resistant to the presence of cyanide were isolated from the cassava-processing wastewater sample. Strains were then named by numbering from N1 to N16 for and kept in stock at -80˚C for further assays.

Figure 10. Selective media plate inoculated with 100uL of cassava-processing effluent and incubated at 30˚C for 5days.



5.3 GRAM STAIN AND OPTICAL MICROSCOPY

Gram stain technique is a cellular characterization based on the ability of the cells in retaining the crystal violet dye due to the presence of specific structures in the cell wall of the microorganism. Gram stain and optical microscopy characterization are the first steps to identify a microbial strain. Gram-positive possess a thick cell wall compared to Gram-negative bacteria that have a thinner cellular wall and at the same time, possess an additional outer

membrane, which gives cells different characteristics when responding to stress, heat, or antibiotics (MAI-PROCHNOW et al., 2016).

Each isolated strain was Gram-stained and analyzed by optical microscopy to identify cell morphology and classify each as Gram-positive or Gram-negative organisms. Pictures taken during analysis are shown in Figure 11 and Figure 12, and Table 9 shows the results of strain characterization. Optical microscopy images were all obtained at the magnification. Strains N1, N4, N5, N6, N7, N8, N9, N10, and N11 were classified as Gram-negative bacteria. Strains N2, N3, N12, N13, N14, N15, and N16 were classified as Gram-positive organisms. All isolated organisms presented a rod-shape cell morphology with little difference in size and shape.

Figure 11. Gram stain of individual strains N1-N8 after cultivation in LB medium and incubation at 30˚C for 24 hours.

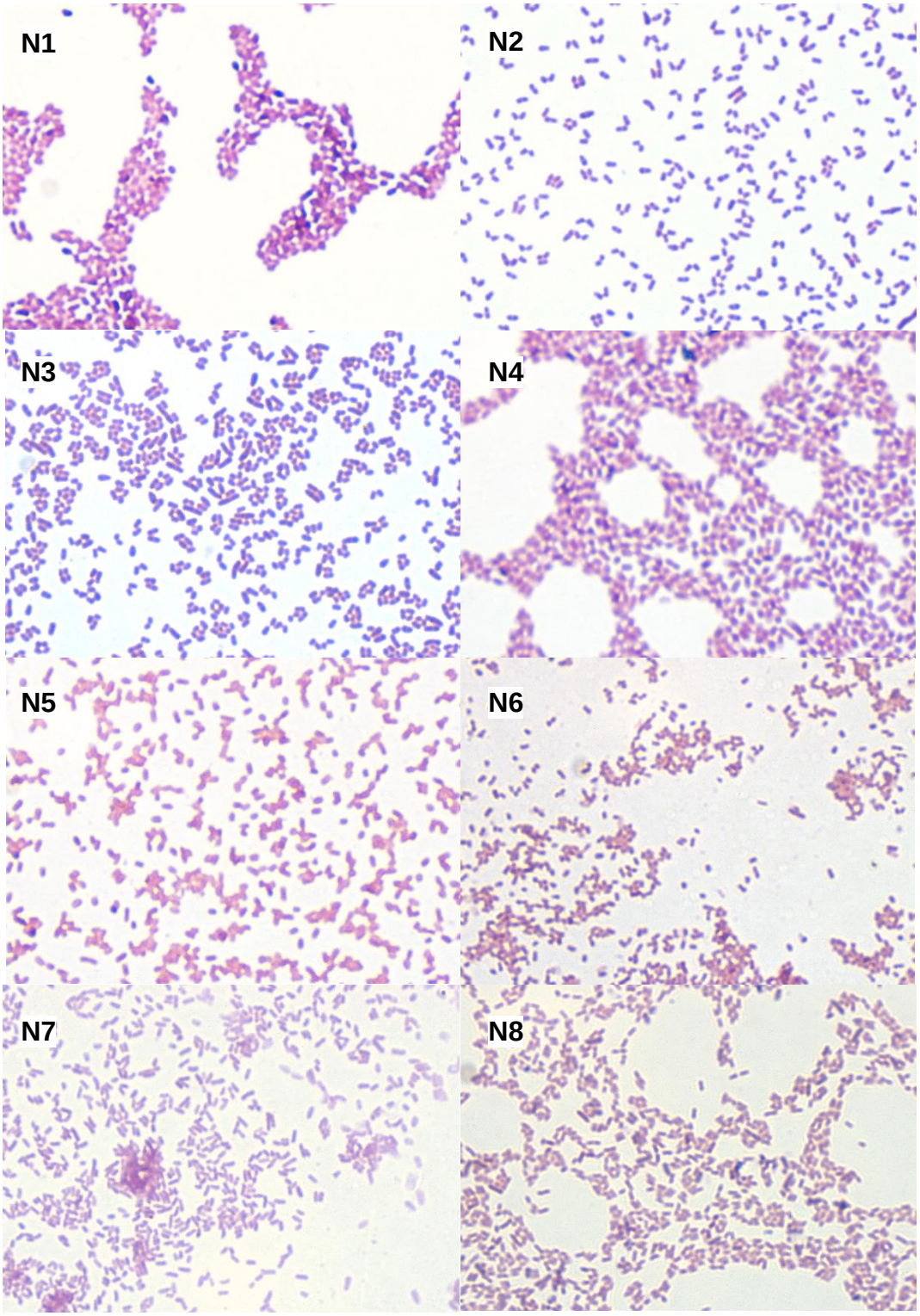


Figure 12. Gram stain of individual strains N9-N16 after cultivation in LB medium and incubation at 30˚C for 24 hours.

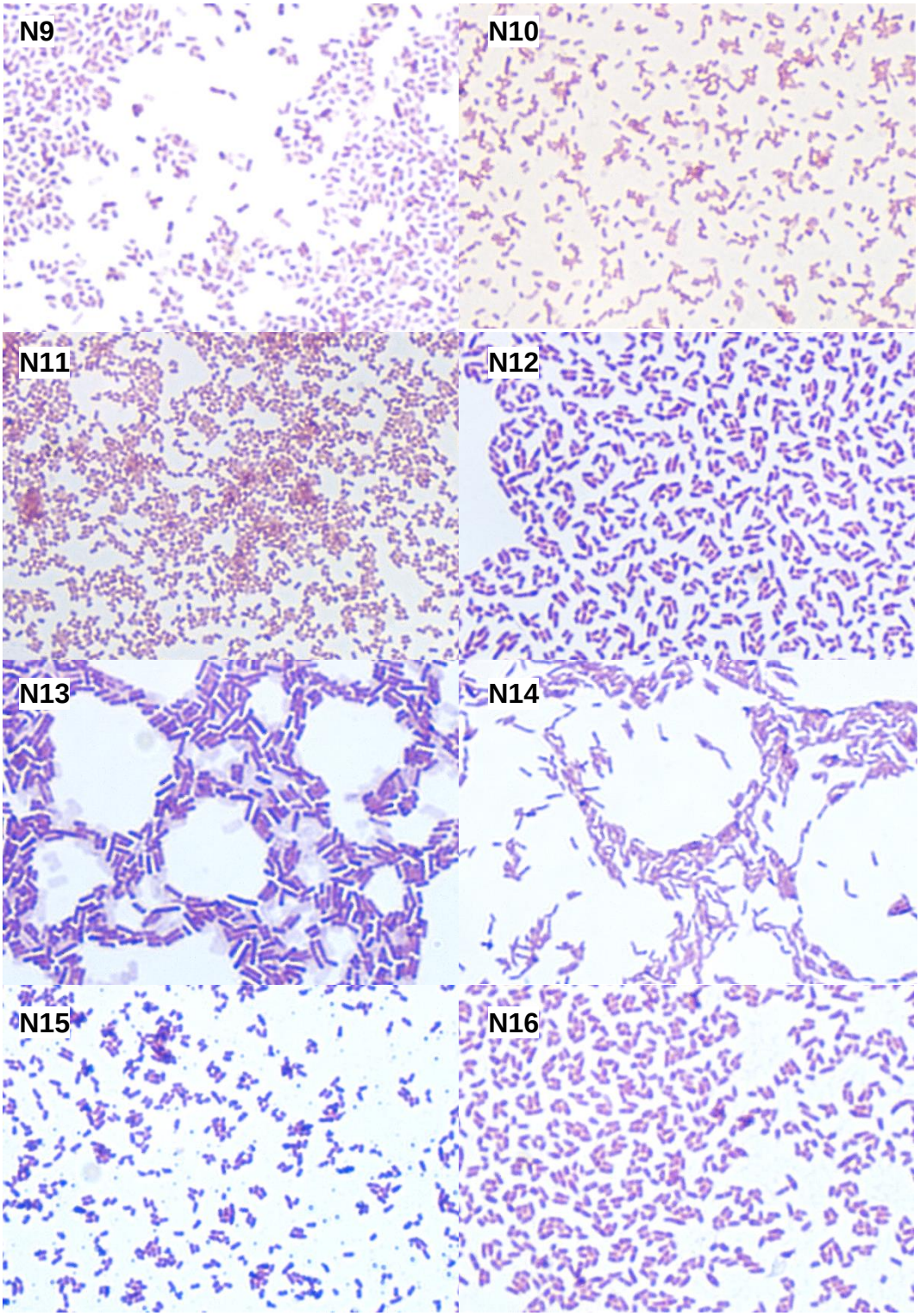


Table 9. Gram and morphological characterization of isolated strains

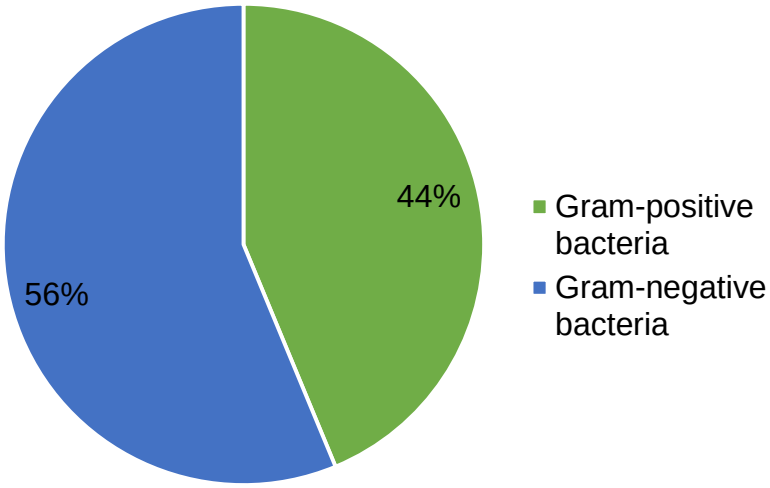
Sample	Gram stain	Cell Morphology
N1	-	Rod-shape
N2	+	Rod-shape
N3	+	Rod-shape
N4	-	Rod-shape
N5	-	Rod-shape
N6	-	Rod-shape
N7	-	Rod-shape
N8	-	Rod-shape
N9	-	Rod-shape
N10	-	Rod-shape
N11	-	Rod-shape
N12	+	Rod-shape
N13	+	Rod-shape
N14	+	Rod-shape
N15	+	Rod-shape
N16	+	Rod-shape

In general, from the 16 strains isolated from the cassava-processing wastewater, 56% were classified as Gram-negative and 44% were classified as Gram-positive (Figure 13). Commonly, Gram-positive bacteria are considered to be more resistant than Gram-negative bacteria even when Gram-negative cells have an extra layer of lipopolysaccharide (LPS) before toxic compounds penetration that Gram-positive bacteria do not possess, although Gram-positive bacteria can increase their resistance to environmental conditions when presenting spores (FALKIEWICZ-DULIK; JANDA; WYPYCH, 2015).

Bacteria have been previously reported as cyanide-degrading organisms, including Gram-positive species belonging to the *Bacillus* and *Corynebacterium* genera (LEE et al., 2003; GUPTA; BALOMAJUMDER; AGARWAL, 2010; MEKUTO et al., 2013; WU et al., 2014), and Gram-negative species belonging

the *Pseudomonas* (WATANABE et al., 1998; LUQUE-ALMAGRO et al., 2005a, 2005b; SINGH; BALOMAJUMDER, 2016), *Burkholderia* (ADJEI; OHTA, 1999), *Klebsiella* (KAO et al., 2003; CHEN; KAO; CHEN, 2008), and *Enterobacter* (MEKUTO; NTWAMPE; AKCIL, 2016) genera.

Figure 13. Distribution of Gram-positive and Gram-negative strains in autochthonous microorganisms isolated from a cassava-processing effluent



5.4 MICROORGANISMS IDENTIFICATION

The 16 isolated strains were identified using a MALDI-TOF mass spectrometry technique. Identification assays were performed from fresh cultures in LB solid medium and in triplicates. Protein spectra obtained for each strain are shown in Annex A. Table 10 shows the best score obtained for each strain. Isolates were identified by their protein spectrum which acts as a fingerprint for each species and obtains protein spectra are compared to data previously identified and are scored from 0 to 3 according to Table 7.

Strains N1, N4, and N5 were classified as *Klebsiella oxytoca* strains. Results for strains N1 and N4 show secure identification for genus and probable species identification, meanwhile strain N5 showed a score above 2.3 indicating a high probable species identification. *Klebsiella oxytoca* is a facultative bacteria, it has been previously isolated from cyanide-containing wastes and it has been reported its ability to degrade cyanide into ammonia (KAO et al., 2003; LEE et al., 2003; CHEN; KAO; CHEN, 2008).

Table 10. Strain identification by MALDI-TOF mass spectrometry technique

Sample	Identification by MALDI-TOF	Score
N1	*Klebsiella oxytoca*	2.298
N2	*Corynebacterium glutamicum*	2.135
N3	*Corynebacterium glutamicum*	1.936
N4	*Klebsiella oxytoca*	2.288
N5	*Klebsiella oxytoca*	2.494
N6	*Serratia marcescens*	2.377
N7	*Enterobacter asburiae*	2.338
N8	*Enterobacter kobei*	2.27
N9	*Serratia marcescens*	2.379
N10	*Serratia marcescens*	2.305
N11	*Enterobacter cloacae*	2.265
N12	*Corynebacterium glutamicum*	2.12
N13	*Bacillus pumilus*	2.012
N14	*Cellulosimicrobium cellulans*	1.897
N15	*Corynebacterium glutamicum*	2.052
N16	*Corynebacterium glutamicum*	1.782

Protein spectra for strains N2, N3, N12, N15, and N16 demonstrated that they belong to the *Corynebacterium glutamicum* species. However, this identification is only secure at the genus level, because the score achieved by strains were between 2 and 2.299. *Corynebacterium glutamicum* species are used industrially to produce several amino acids and has been reported to have a cyanide-resistant bypass oxidase pathway in its respiratory chain (MATSUSHITA et al., 1998). Bacteria belonging to the *Corynebacterium* genus is also known to be an important lactic acid bacteria (LAB), which are microbial strains associated with the production of linamarase during fermentation of cassava (BEHERA; RAY, 2017).

Strains N6, N9, and N10 were identified as *Serratia marcescens* species. All three strains achieved a highly possible identification at the species level. *Serratia marcescens* has been previously reported as a cyanide-degrading bacteria in a study performed in Malaysia, isolated from soil and drainage water samples (KARAMBA et al., 2015) and then optimized for cyanide degradation using glucose and yeast extract as nutrients sources, incubated at 32.5˚C, pH 6 and with 200mg.L^{-1} of initial cyanide concentration (KARAMBA et al., 2016). Another study showed that cyanide degradation by *Serratia marcescens* species forms formamide at the end which evidences the cyanide hydratase enzymatic pathway (KUSHWAHA et al., 2018).

Strains N7, N8, and N11 were identified as *Enterobacter asburiae*, *Enterobacter kobei* and *Enterobacter cloacae*, respectively. Only the molecular identification of the *Enterobacter asburiae* strain (N7), achieved a score above 2.3, categorizing it as highly probable for species identification. Meanwhile, strains N8 and N11 achieved a score between 2 and 2.20 indicating reliable genus identification and probable species identification. Furthermore, protein spectra of strains belonging to the Enterobacter complex are very similar and it is difficult to distinguish between species (BRUKER DALTONICS, 2015b). Therefore, strains N7, N8 and N11 can be classified as *Enterobacter* spp. Cyanide-tolerant strains belonging to the *Enterobacter* genus have been reported to form β-cyano-L-alanine from cyanide degradation (SAKAI et al., 1981). At the same time, *Enterobacter kobei* and *Enterobacter asburiae* have been reported as growth-promoting bacteria which are often found in tubers and maize roots, and which have the characteristic of producing cyanide and ammonia as biological control of plants pathogens (AHEMAD; KHAN, 2010; OGBO; OKONKWO, 2012).

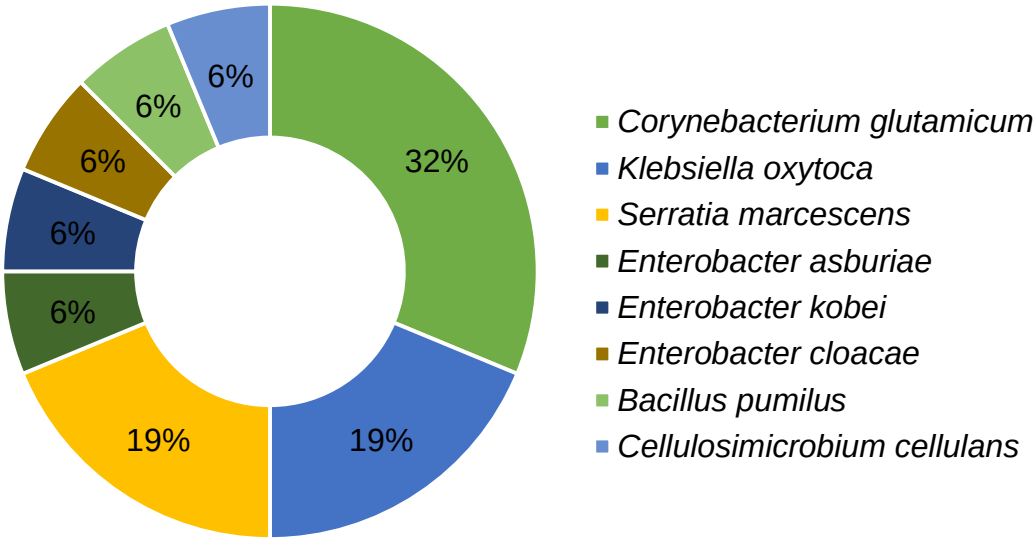
Strain N13 was identified as *Bacillus pumilus* with a score of 2.012 indicating secure genus identification and probable species identification. Several strains belonging to the *Bacillus* genus have been studied in the degradation of cyanide individually (SKOWRONSKI; STROBEL, 1969; WU et al., 2014; KANDASAMY et al., 2015; ROSARIO, 2017) and in consortia (MEKUTO et al., 2013; NWOKORO; DIBUA, 2014; RAZANAMAHANDRY et al., 2016). *Bacillus pumilus* has been reported to use a cyanidase enzyme to transform cyanide in

ammonia and formate (JANDHYALA et al., 2003) and has been adapted to degrade cyanide in alkaline conditions (ROSARIO, 2017).

Results for strain N14 registered a score of 1.897 identified as a *Cellulosimicrobium cellulans* strain while showing a probable genus identification according to its similarity to protein spectra data. *Cellulosimicrobium cellulans* has been identified as a possible plant growth-promoting bacteria (PGPB) isolated from soils (NABTI et al., 2014) and therefore, it is possible to be found together with the other isolated strains in this study. However further analysis using a 16s technique would be needed to identify the species of this strain with more reliability.

From Figure 14, we can observe the distribution of identified species of the 16 isolates from cassava-processing wastewater. *Klebsiella oxytoca*, *Corynebacterium glutamicum*, and *Serratia marcescens* were the species found in the effluent with more frequency, with 19%, 32%, and 19% respectively. Most of the times, strains together in a hostile environment develop symbiosis relationships to survive (RAZANAMAHANDRY et al., 2016). Moreover, these relationships can be sometimes dependent and therefore, they can explain why sometimes microorganisms in consortia are able to degrade toxic compounds that are not able to degrade individually.

Figure 14. Distribution of isolated cyanide-resistant strains from cassava-processing wastewater.

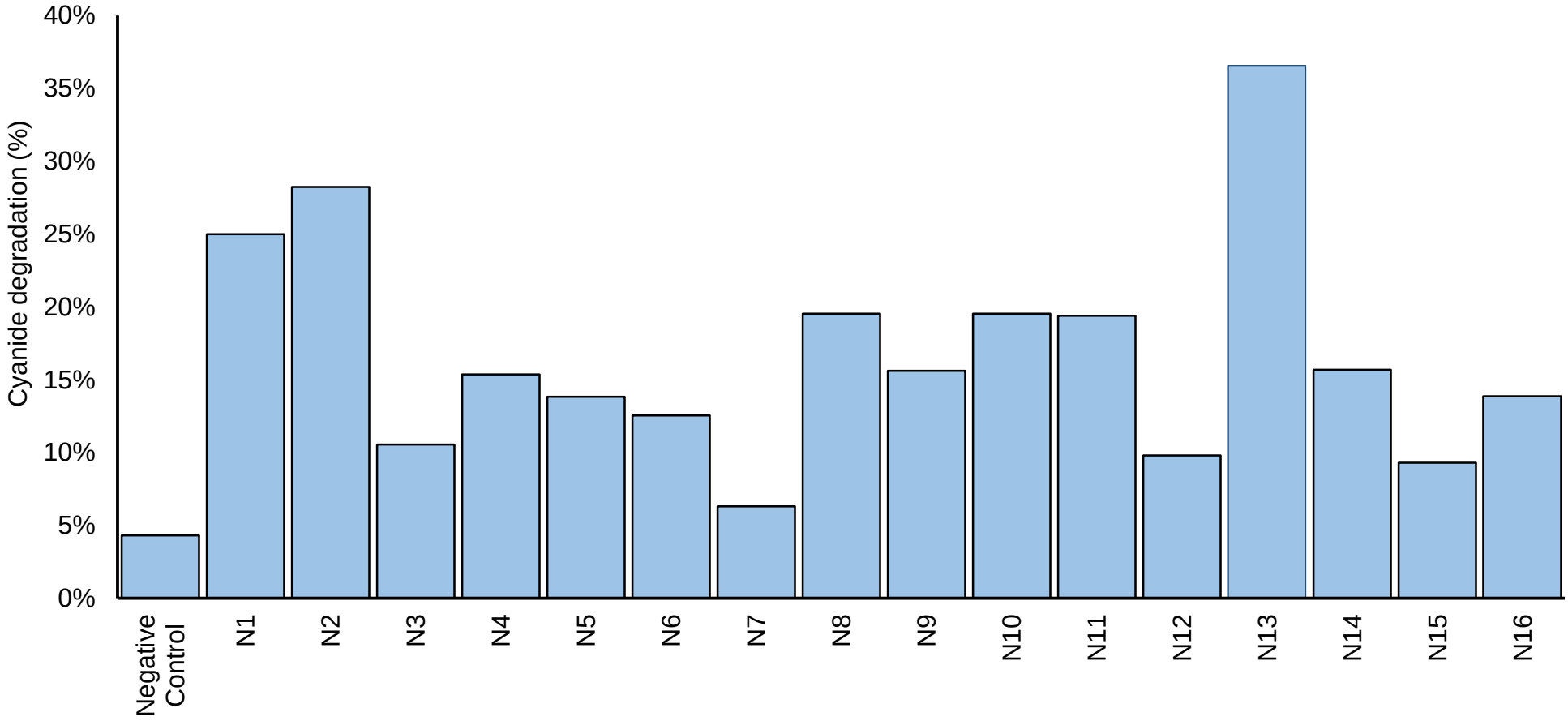


5.5 CYANIDE DEGRADATION SCREENING

Strains were individually tested for free-cyanide degradation with the aim to select the bacteria with more degradation potential for further assays. As most enzymatic pathways for cyanide degradation do not synthesis biomass, it was important to perform the assessment with an initial concentration of cell of OD=1 that indicated the late exponential growth of the strain and where they usually express all of their enzymes (KAEWKANNETRA et al., 2009).

Figure 15 shows the degradation rate for each isolated strain after 16 hours of incubation at 30ºC and 185RPM. The assay was performed in triplicates and the average between triplicates was shown as the result. A negative control containing no bacteria was incubated at the same condition to evaluate cyanide volatilization and natural degradation.

Figure 15. Cyanide-degrading screening by isolated strains after 16h of incubation in orbital agitation at 185RPM 30°C. Cyanide degradation (%) from a minimum media containing 150mg.L^{-1} CN^- at pH8.



As can be seen in the green bar of the graph, the negative control presented a degradation of less than 4.3%. This degradation occurs due to the pH of the solution being lower than the pKa of cyanide and due to cyanide natural degradation explained previously by Equation 3. Therefore, it is assumed that the difference in the degradation rates in all of the other samples is a result of the action of bacteria.

The strain that presented the lowest degradation rate was N7, identified as *Enterobacter asburiae*, which showed a degradation rate of 6.3%. As explained before, this species has been reported as a PGPB, a group of bacteria that are normally present in plants root who have the characteristic of generating small amounts of cyanide that act as a defense for plant pathogens (AHEMAD; KHAN, 2010; OGBO; OKONKWO, 2012). Hence, it is within the expected results for these strains to not have developed enzymatic routes for cyanide degradation.

Furthermore, comparing the obtained degradation rates with previous individual identification, it is observed that even strains of the same identified species can show different degradation potential. Identified *Corynebacterium glutamicum* strains showed degradation rates of 28.2%, 10.5%, 9.8%, 9.3% and 13.8%. This variability can be explained by genetic variability or adaptation to environments, since *Corynebacterium glutamicum* strains have been reported to have a cyanide-resistant respiration route but not necessarily a cyanide-degrading enzymatic pathway (MATSUSHITA et al., 1998).

The strain that presented the highest degradation rate was N13, identified as *Bacillus pumilus*, which decreased cyanide concentration by 36.6% in the incubation period. *Bacillus pumilus* has been widely studied for cyanide degradation and its degrading enzyme, cyanidase (also known as CynD), in the transformation of cyanide to ammonia and formate (JANDHYALA et al., 2003).

Other strains that presented high degradation rates were strains N2, N1, N10, N8, and N11, which attenuated free-cyanide concentration by 28.2%, 25%, 19.5%, 19.5%, and 19.3%, respectively. These strains, together with N13, showed the biggest potential to be used in cyanide treatment. Four strains were thus selected to be assessed in their susceptibility to degrade cyanide in hostile conditions. Strains selected were N1, N2, N11, and N13 previously identified as

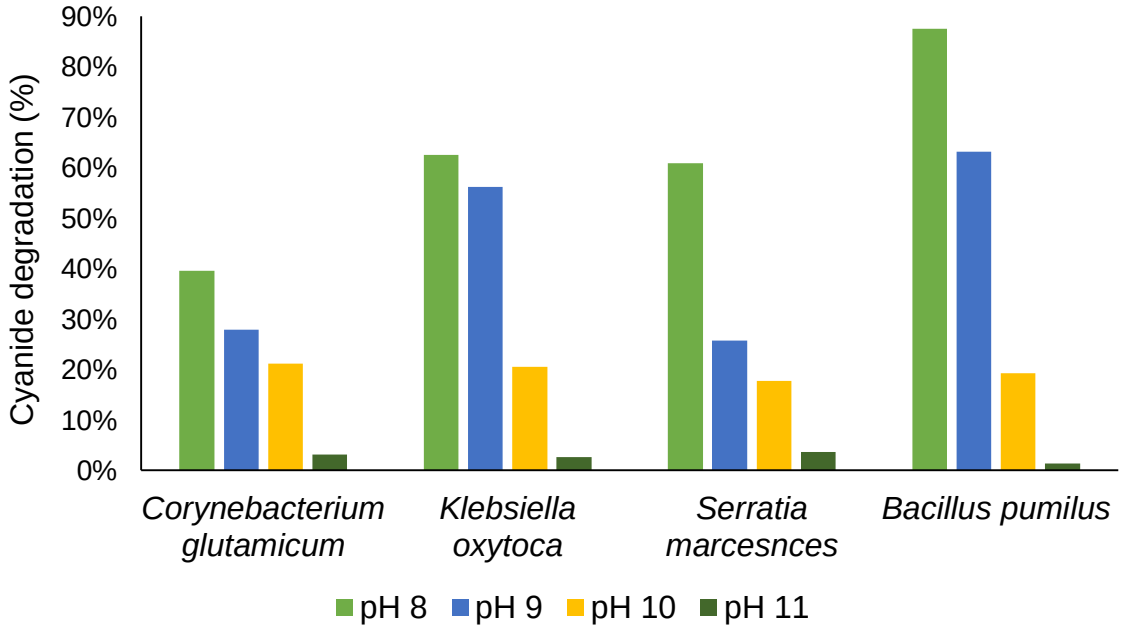
Klebsiella oxytoca, *Corynebacterium glutamicum*, *Serratia marcescens,* and *Bacillus pumilus*, respectively.

5.6 SUSCEPTIBILITY ASSAYS

Microorganisms are able to adapt to hostile environments, including acid, alkaline environments and high concentration of pollutants. However, their metabolism can be affected by environmental conditions including pH, temperature, presence or absence of oxygen, and pollutant concentration (HATZINGER; KELSEY, 2005; JØRGENSEN, 2008). Bacteria can reproduce and mutate very fast. Therefore, depending on the intrinsic characteristics of the species and the specific strain, some organisms can adapt relatively faster or easier to hostile environments than others.

Meanwhile, in cyanide-containing effluents is important to keep high pH values to minimize HCN volatilization during treatment. Therefore, it is important that microorganisms used in a potential biological treatment keep their degradation capacity in alkaline conditions. Susceptibility to pH values for the four selected strains is shown in Figure 16. Results show that higher pH values tend to inhibit cyanide degradation capacity of the strain.

Figure 16. Susceptibility to pH for selected strains after 48h of incubation at 185RPM 30˚C. Cyanide degradation (%) from minimum media containing 150mg.L^{-1} CN^{-}.



Degradation rates after degradation assays incubated for 48hours at pH8, were 39.5%, 62.5%, 60.9%, and 87.5% for *Corynebacterium glutamicum*, *Klebsiella oxytoca*, *Serratia marcescens,* and *Bacillus pumilus* strains, respectively. When the assay was performed at pH9, degradation rates decreased by 11.6%, 6.4%, 35.2%, and 24.4%, respectively, showing that *Klebsiella oxytoca* and *Bacillus pumilus* strains were able to keep their degradation potential at this pH value by achieving a greater degradation rate than the other two studied strains. *Corynebacterium glutamicum* strain presented less variability with pH increase than the other strains; however, total degradation rates were still lower than other native microorganisms. All strains showed a degradation rate below 20% when the assay was performed at pH10 and below 4% when the assay was performed at pH11 showing that higher pH values inhibit the cyanide-degrading ability of isolated strains.

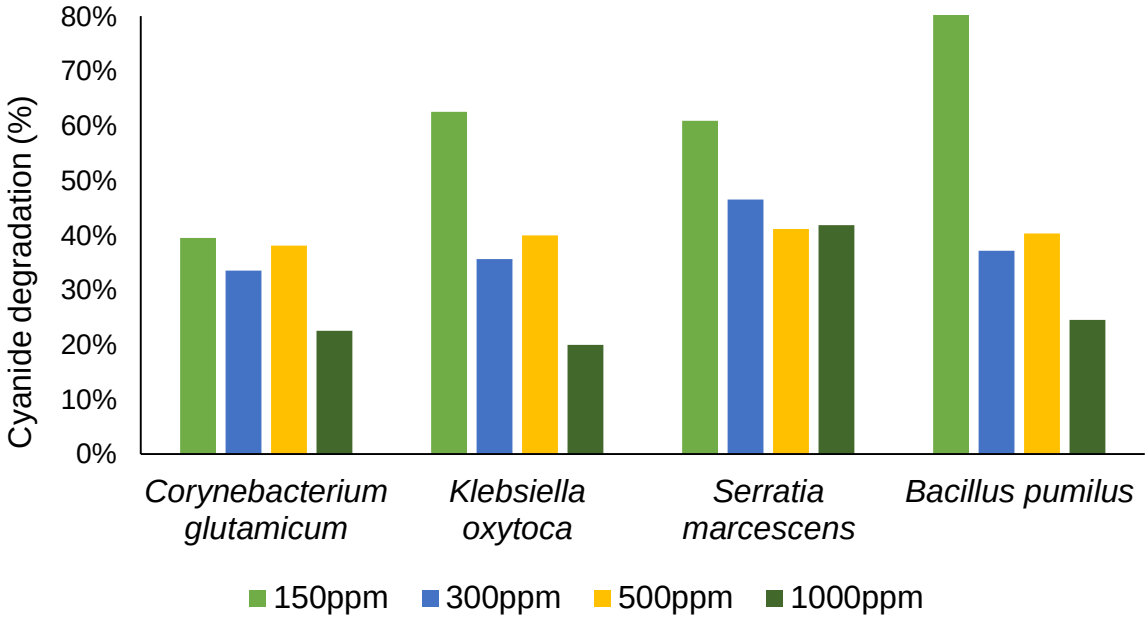
Biological degradation of cyanide in alkaline solutions has been a constant challenge in the application of biotechnologies for treating cyanide. Although a constant pH above 10 makes cyanide less volatile, it also makes cyanide degradation less efficient, or even impossible when high concentrations of cyanide are used (LUQUE-ALMAGRO et al., 2005b; HUERTAS et al., 2010), as most enzymatic activity and bacterial growth are dependent on pH (CRUM; TREVOR SEWELL; BENEDIK, 2016).

Since strains were isolated from cassava-processing wastewater that was at pH5.5, it is reasonable that autochthonous strains are adapted to neutral or slightly acid pH values. Other native isolated strains have also shown difficulties in keeping enzymatic activity when forced to degrade cyanide in alkaline conditions (SANKARANARAYANAN; GOWTHAMI, 2015). In order to use these strains in alkaline cyanide degradation, a slow and gradual adaptation to alkaline environments must be conducted to promote cell activity at pH>10 as performed by (ROSARIO, 2017).

On the other hand, high cyanide concentrations can be toxic even for cyanide degrading microorganisms (KANDASAMY et al., 2015). Figure 17 shows degradation rates for selected strains after a 48hours incubation period at 185RPM 30˚C. *Klebsiella oxytoca*, *Serratia marcescens,* and *Bacillus pumilus* showed greater degradation potential with 62%, 60% and 88% each, respectively

when free-cyanide initial concentration was 150mg.L^{-1}. Higher cyanide concentrations of 300, 500 and 1000mg.L^{-1} caused a decrease in degradation rates. Even though, all tested strains were still able to degrade cyanide in initial concentrations up to 1000mg.L^{-1}.

Figure 17. Susceptibility to initial cyanide concentration for selected strains after 48h of incubation at 185RPM 30˚C. Cyanide degradation (%) from minimum media containing 150mg.L^{-1} CN^- at pH8.



Bacillus pumilus was the strain that showed the greatest susceptibility to initial cyanide concentration increase. Previously reported *Bacillus sp.* and *Klebsiella sp.* strains could only tolerate cyanide concentrations up to 650mg.L^{-1} (SANKARANARAYANAN; GOWTHAMI, 2015) and up to 750 mg.L^{-1} for *Serratia marcescens* (KARAMBA et al., 2016), which means that isolated strains show potential for biotreating cyanide even in high concentrations.

Corynebacterium glutamicum strains showed less variability when changing initial cyanide concentration. Nevertheless, the degradation capacity of this strain is commonly lower than the other species assessed in this study.

Susceptibility assays give essential information for strain selection. A strain with less susceptibility will have a greater potential to be used in different treating conditions. Conducted experiments showed that from the four assessed organisms, isolated *Klebsiella Oxytoca* and *Bacillus pumilus* strains had less susceptibility to pH change and therefore show a bigger potential to treat cyanide

in alkaline conditions, whereas isolated *Klebsiella oxytoca*, *Serratia marcences,* and *Bacillus pumilus* strains presented higher degradation rates when initial cyanide was 150mg.L^{-1} even when they were still able to degrade cyanide in initial concentrations up to 1000 mg.L^{-1}.

From the selected bacteria, *Bacillus pumilus* strain has been assessed separately by another study (ROSARIO, 2017) and therefore, will not be considered in the scope of this work. Consequently, isolated strains of *Klebsiella oxytoca* and *Serratia marcescens* were selected for deeper study in their potential use for cyanide degradation, because they showed a higher potential during susceptibility assays.

5.7 BACTERIAL GROWTH ANALYSIS

Bacterial growth for selected strains was assessed in order to understand the dynamics of microbial populations during the degradation process. Growth curves were obtained measuring optical density at λ=600 (OD_{600}) and using the Miles and Misra method for viable cell counting for 24hours. The results of this are shown in Figure 18 and Figure 19 for *Serratia marcescens* and *Klebsiella oxytoca* strains, respectively.

Figure 18. Growth curve for isolated *Serratia marcescens* strain in LB medium incubated at 185RPM 30°C.

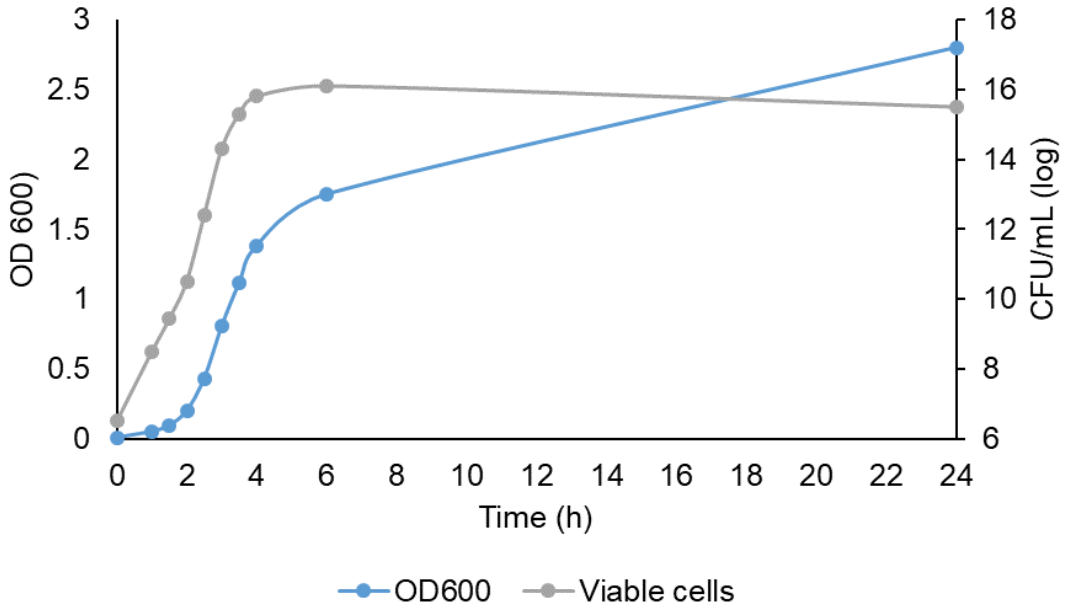
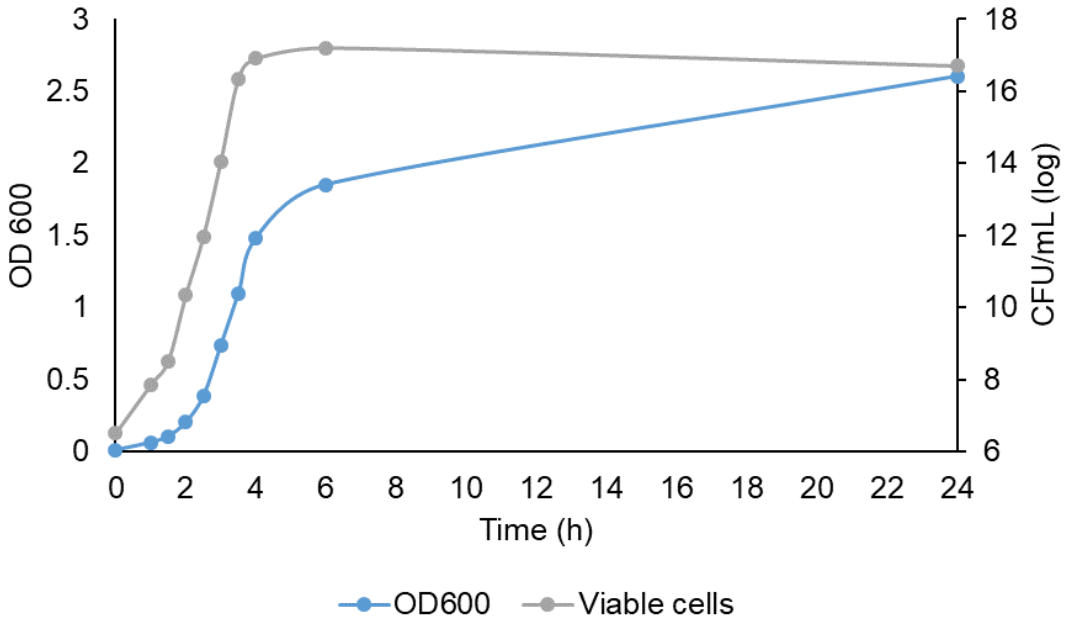


Figure 19. Growth curve for isolated *Klebsiella oxytoca* strain in LB medium incubated at 185RPM 30°C.



Bacterial growth curves allowed us to understand the kinetics of microorganisms' growth and helped to understand the phases of the process in order to choose the stage of the culture in which the cells will be harvested for biological treatment. Furthermore, growth curves helped to understand the relationship between the optical density (OD_{600}) and the culture density, measured in the viable cells per volume ($CFU.mL^{-1}$).

For both strains, the exponential growth phase took place between the first and fourth hours of incubation. Both strains reached their stationary phase of growth after 5hours of incubation at 30°C and 185RPM.

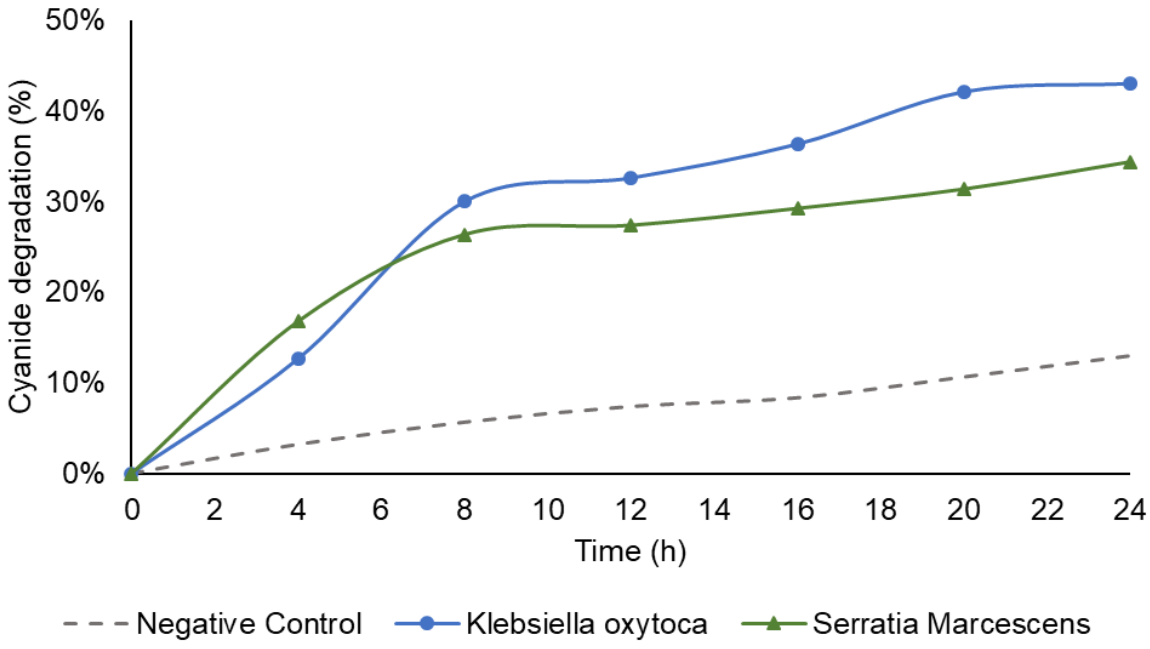
The isolated strain of *Serratia marcescens* began its exponential growth curve after 1hour of incubation at OD_{600}=0.05, culture density of $10^{8.5}CFU.mL^{-1}$, and reached its stationary phase at OD_{600}=1.4 and culture density of $10^{15.8}CFU.mL^{-1}$. For the isolated strain of *Klebsiella oxytoca*, the exponential growth phase started after 1hour of incubation at OD_{600}=0.06 and culture density of $10^{7.8}CFU.mL^{-1}$ and reached its stationary phase at OD_{600}=1.5 and culture density of $10^{16.9}CFU.mL^{-1}$.

Because of this, it was decided that both cultures will be harvested at OD_{600}=0.8 as it indicates the middle of the exponential growth phase and therefore, the moment where the cultures will be duplicating their biomass per unit of time, showing a constant growth rate and uniform metabolic activity (PARKER et al., 2018). Harvesting cells at this optical density would mean a cell concentration of approximately 10^{10}CFU.mL^{-1} for each culture.

5.8 DEGRADATION RATES ASSESSMENT

The study of free-cyanide degradation by isolated strains was conducted in order to study the rates of cyanide degradation in a 24h period. Individual strains were assessed and compared to a negative control containing no bacteria. Residual cyanide was measured and cyanide degradation was calculated for each time. Figure 20 shows cyanide degradation by individual strains of *Klebsiella oxytoca* and *Serratia marcescens* using cyanide as a whole nitrogen source and in the presence of minimum salts at pH8. In the same figure, negative control showed the natural degradation of cyanide to be lower than 10% for the assessed period showing the actual benefit of using native cyanide-degrading bacteria for biotreatment.

Figure 20. Cyanide degradation by isolated strains at 185RPM 30°C



The assay solution presented an initial concentration of 150mg.L^{-1} of free cyanide. Isolated *Klebsiella oxytoca* strain degraded 43% of cyanide after the 24h incubation period, residual cyanide concentration in solution was of 85mg.L^{-1}. Meanwhile, *Serratia marcescens* strain achieved a 34% degradation of free-cyanide under the same culture and incubation conditions leaving a residual cyanide concentration of 98mg.L^{-1}. In these results, we can observe that there is a difference between the negative control and the individual strain treatment. Individual strains could increase cyanide degradation by up to 32% more indicating the potential use of these strains in cyanide biotreatment. However, they were not able to degrade cyanide completely or down to non-toxic concentrations during the evaluated period.

Cyanide degradation occurred at a 2.68mg.L^{-1}.h^{-1} and 2.15mg.L^{-1}.h^{-1} for *K. oxytoca* and *S. marcescens* strains, respectively. Higher degradation rates were observed during the first 4hours of incubation for *Serratia marcescens*, with a degradation rate of 6.28mg.L^{-1}.h^{-1} and between the 4^{th} and 8^{th} hour of incubation for the *Klebsiella oxytoca* strain with a cyanide degradation rate of 6.5mg.L^{-1}.h^{-1}. Then, in both cases, degradation rates decrease to near 1mg.L^{-1}.h^{-1}.

During cyanide degradation assays, pH and cell concentration were measured to monitor the microbial growth and to confirm that the pH conditions remained the same during the incubation period for reducing the cyanide volatilization risk and keeping physiological conditions for bacteria. Figure 21 and Figure 22 show the process monitoring for *Klebsiella oxytoca* and *Serratia marcescens*, respectively. In both cases, pH values are maintained between 7 and 8, assuring neutral values that would allow bacteria to develop normally and organisms would be able to use their cyanide-degrading metabolic pathways.

Figure 21. Free-cyanide degradation by the isolated strain of *Klebsiella oxytoca*. Viable cell and pH monitoring. Incubation at 30ºC 185RM.

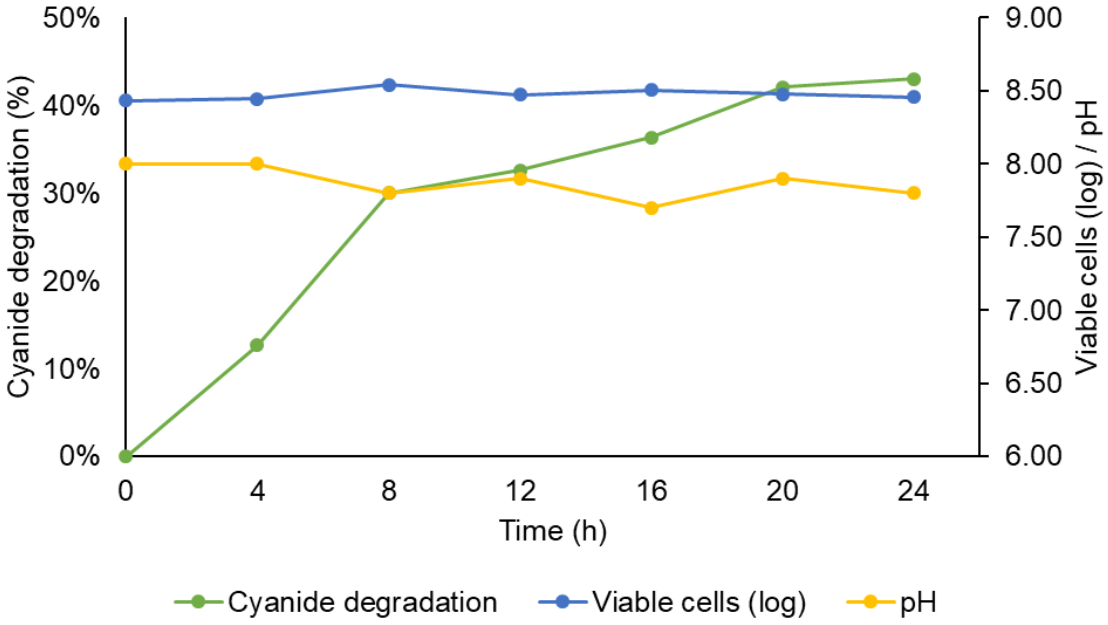
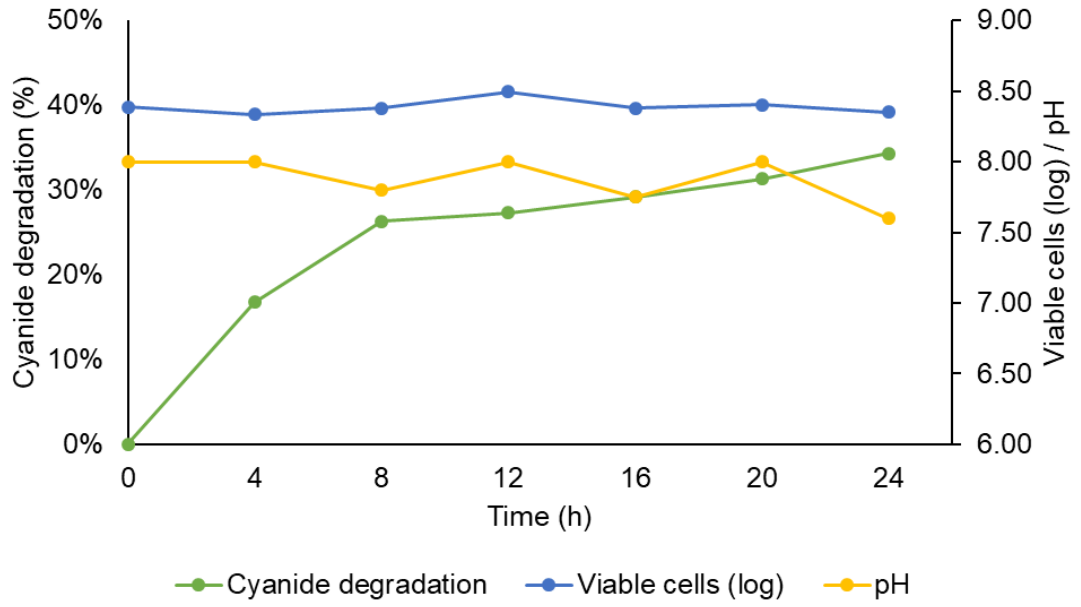


Figure 22. Free-cyanide degradation by the isolated strain of *Serratia marcescens*. Viable cell and pH monitoring. Incubation at 30ºC 185RM.



Culture density of both strains is also seen to remain fairly constant through the incubation period at a cell concentration of approximately 10^8CFU.mL^{-1}. This means, that viable cells number are not increasing even if

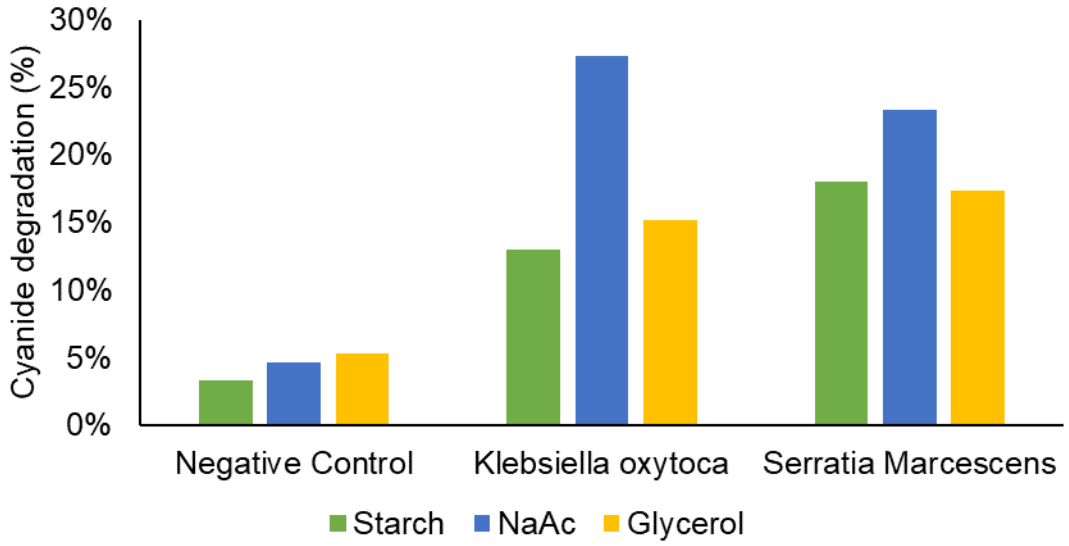
cyanide is being degraded. Furthermore, cell population doesn't decrease either during the assessed period which could mean that both strains are consuming cyanide in metabolic pathways that not necessarily lead to the synthesis of new biomass but rather biotransform cyanide through nongrowth processes called cometabolism. Cometabolic reactions transform the chemical structure of pollutants but cells do not gain energy, carbon or inorganic nutrients directly from the reaction, which can also occur when substrate reacts with enzymes with a broad substrate specificity (HATZINGER; KELSEY, 2005). This can later result in the production of other intermediates that can be later mineralized or used in other metabolic routes by microorganisms.

5.9 OPTIMIZATION OF CYANIDE DEGRADATION

5.9.1 INFLUENCE OF CARBON SOURCE

An easily metabolizable carbon source is needed for cyanide degradation since microorganisms can use the nitrogen from CN molecules but not the carbon (MIRIZADEH; YAGHMAEI, 2014). Cyanide may react with aldehydes and ketones (i.e. glucose) to form ammonia abiotically and therefore, biological loss of cyanide can be masked depending on media composition (ADJEI; OHTA, 1999; LUQUE-ALMAGRO et al., 2005b). Figure 23 shows cyanide degradation by *K. oxytoca* and *S. marcescens* strains using starch, sodium acetate, and glycerol as carbon sources. All negative controls showed 5% cyanide degradation or less after 16h incubation. For the isolated *Klebsiella oxytoca* strain, the highest cyanide degradation rate was achieved using sodium acetate as a carbon source, followed by glycerol and starch as carbon sources, respectively. Meanwhile, the isolated *Serratia marcescens* strain achieved the highest degradation rate also using sodium acetate as a carbon source followed by starch and then glycerol.

Figure 23. Influence of carbon source in cyanide degradation of isolated strains after 16h incubation at 185RPM 30°C.



During cassava processing, starch is liberated in solution from the tubers, therefore, it was expected for native microorganisms to be able to use this as a carbon source. However, starch is a polysaccharide carbohydrate consisting of several glucose monomers which may be not so easily accessible for individual cellular microorganisms. Sodium acetate has been effectively used as a carbon source for cyanide degradation by previous authors (LUQUE-ALMAGRO et al., 2005b; MIRIZADEH; YAGHMAEI, 2014). Meanwhile, glycerol has been previously used as a carbon source in cyanide-degrading isolating media (WU et al., 2014). According to WU et al. (2014), the addition of glycerol could promote the growth of *Bacillus sp.* degrading bacteria.

In this study, assay media containing sodium acetate as carbon source resulted in the highest degradation percentage for both strains, achieving 27 and 22% for *Klebsiella oxytoca* and *Serratia marcescens* isolated strains, respectively. Therefore, the following assays used sodium acetate as a carbon source for cyanide degradation.

5.9.2 INFLUENCE OF TEMPERATURE INCUBATION

Results for cyanide degradation by isolated *Klebsiella oxytoca* strain are shown in

Figure 24. After a 24h incubation period, it was observed that *Klebsiella oxytoca* strain achieved a higher cyanide degradation percentage at 30°C. With incubation at this temperature, the strain could reduce initial cyanide concentration by 43% after 24 hours. When lower temperatures of 25°C and 20°C were tested, cyanide degradation was lower, achieving 31 and 21% of degradation, respectively.

Figure 24. Cyanide degradation by isolated *Klebsiella oxytoca* strain from a 150mg.L^{-1} free-cyanide synthetic solution. Incubation for 24h at 185RPM and at 20, 25 and 30°C.

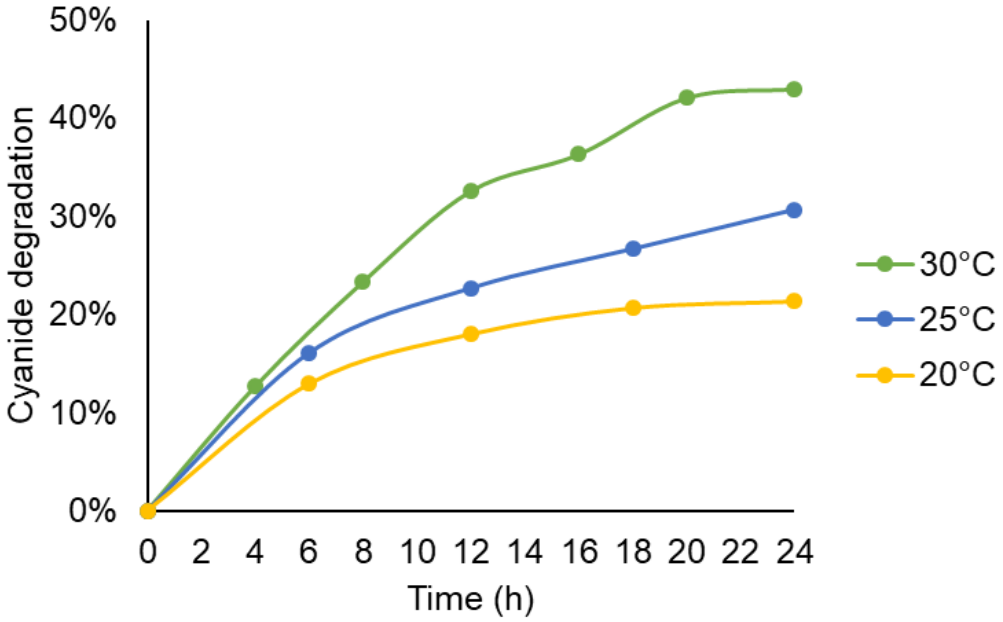
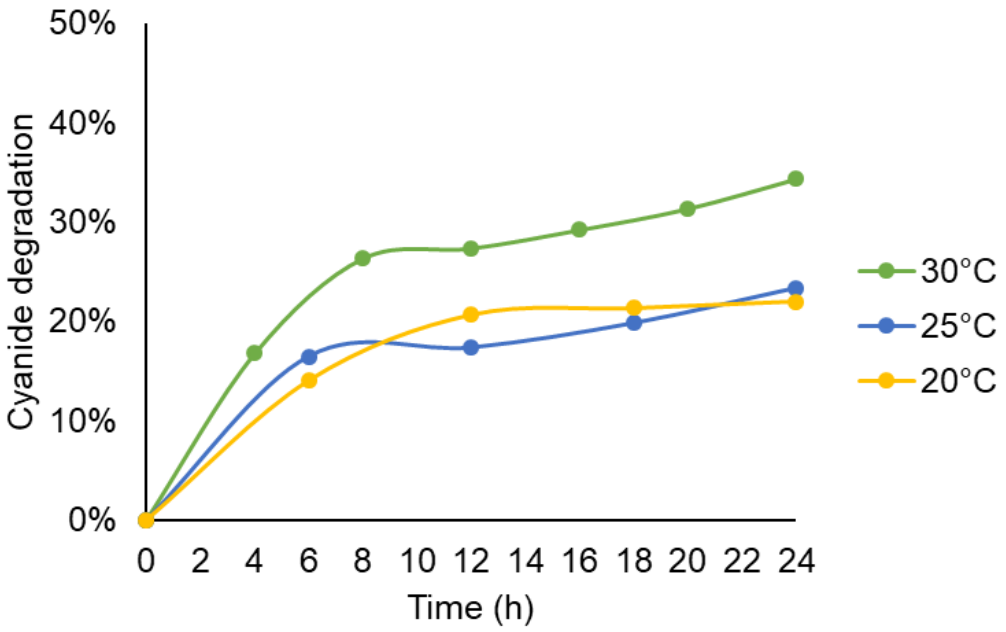


Figure 25 shows cyanide degradation by isolated *Serratia marcescens* strain at different temperatures. Results showed that the *Serratia marcescens* strain could remove cyanide at a higher rate when incubated at 30°C achieving 34% of cyanide degradation after a 24h incubation period. When incubated at 25 and 20°C, the *Serratia marcescens* strain was able to degrade 23 and 22%, respectively.

Figure 25. Cyanide degradation by isolated *Serratia marcescens* strain from a 150mg.L^{-1} free-cyanide synthetic solution. Incubation for 24h at 185RPM and at 20, 25 and 30°C.



Temperature influence in cyanide degradation has been point out as one of the most determining factors for degradation optimization because of the sensitivity of cyanide-degrading enzymes that need a suitable temperature (WU et al., 2014). Overall, results showed a higher cyanide removal for both strains when incubated at 30°C. This indicates that a biotreatment process at environment temperatures would have a lower efficiency than if slightly heated. Previous studies confirm that usually optimum temperature for cyanide degradation is around 30°C, as both of the strains detailed in this study (AKCIL et al., 2003; CHEN; KAO; CHEN, 2008; HUERTAS et al., 2010).

5.9.3 INFLUENCE OF ROTATION SPEED INCUBATION

During cyanide degradation, rotation speed affects the mass transfer of dissolved oxygen, substrate and nutrients from liquid to bacterial surface and helps maintaining uniform conditions in media (GUAMÁN GUADALIMA; NIETO MONTEROS, 2018). In this study, the influence of rotation speed during incubation in the cyanide degradation rate by isolated bacteria was evaluated. Degradation assays incubated at 135, 185 and 235RPM were assessed. Figure

26 and Figure 27 show the results for cyanide degradation under different rotation speed conditions for isolated *K. oxytoca* and *S. marcescens* strains, respectively.

Figure 26. Cyanide degradation by isolated *Klebsiella oxytoca* strain from a 150mg.L^{-1} free-cyanide synthetic solution. Incubation for 24h at 30°C and at 135, 185 and 235RPM.

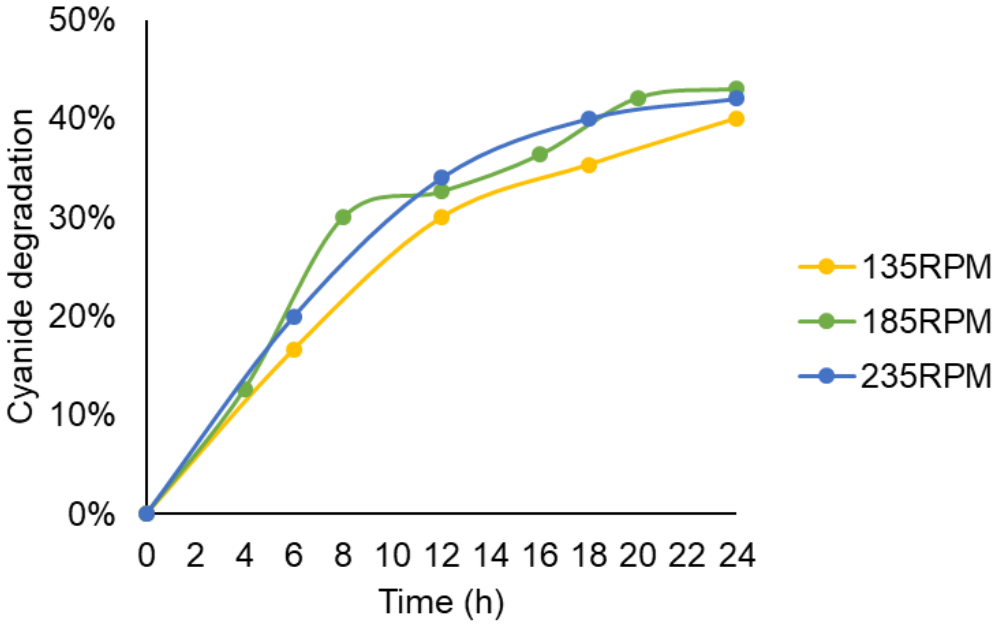
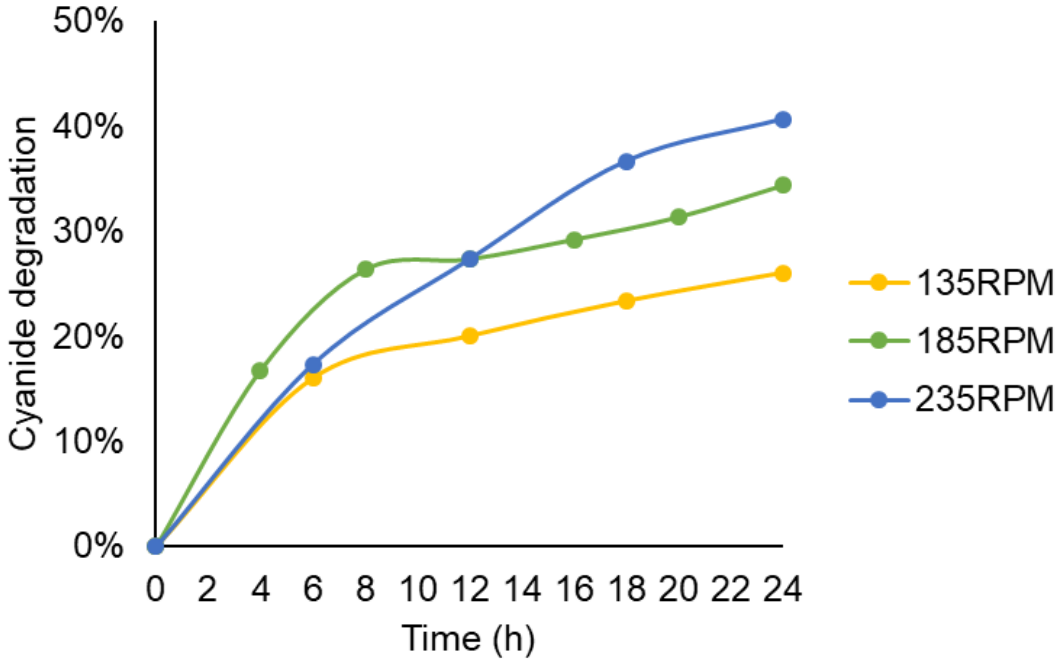


Figure 27. Cyanide degradation by isolated *Serratia marcescens* strain from a 150mg.L^{-1} free-cyanide synthetic solution. Incubation for 24h at 30°C and at 135, 185 and 235RPM.



It was observed that cyanide degradation by isolated *Klebsiella oxytoca* is not influenced by different rotation speeds. Assays at 135, 185 and 235RPM resulted all in similar degradation percentages after a 24h incubation period achieving approximately 40% cyanide removal. Meanwhile, cyanide degradation by isolated *Serratia marcescens*, was observed to be positively favored by agitation speed. The *Serratia marcescens* strain achieved the highest cyanide degradation result when agitated at 235RPM, obtaining 41% removal, and the lowest when agitated at 135RPM, degrading only 26% of free-cyanide in solution after 24hours.

These results indicate that the oxygen supply for the isolated *Klebsiella oxytoca* does not influence in cyanide removal. This behavior may be caused by the reported ability of *Klebsiella oxytoca* species to degrade cyanide in both aerobic and anaerobic environments (KAO et al., 2003; CHEN; KAO; CHEN, 2008). On the other hand, authors have previously analyzed the influence of inoculum size, temperature and pH on the degradation of cyanide by *Serratia marcescens* (KARAMBA et al., 2016); but, to our knowledge, this is the first report on the influence of rotation speed on the degradation of cyanide by *Serratia marcescens.*

After the evaluation of the isolated strains of *Klebsiella oxytoca* and *Serratia marcescens* from a cassava processing effluent, results show the potential use of these strains in cyanide biological removal. Optimal conditions for cyanide degradation by isolated *Klebsiella oxytoca* strains were observed by using sodium acetate as a carbon source and cyanide as a nitrogen source, pH 8 and incubated at 30°C. Speed rotation during the incubation didn't show any effect on cyanide degradation by this strain. Meanwhile, isolated *Serratia marcescens* strains showed higher cyanide removal when using sodium acetate as a carbon source and cyanide as a nitrogen source, pH 8 and incubated at 30°C and 235RPM. Under optimal conditions, both strains achieved a biological degradation of cyanide above 40% of initial concentration, 150mg.L^{-1}

Table 11. Comparative data of free cyanide degradation by *Klebsiella oxytoca* and *Serratia marcescens* under different conditions.

Strain	Carbon source	[CN-]i	pH	T (°C)	Time (h)	Cyanide removal (%)	Reference
Klebsiella oxytoca	Glucose	21	7	30	32	91	(KAO et al., 2003)
Klebsiella oxytoca	Sodium acetate	150	8	30	24	43	This study
Serratia marcescens	Glucose	80	6	32	120	95.6	(KARAMBA et al., 2016)
Serratia marcescens	Sodium acetate	150	8	30	24	34	This study

Results obtained in this study are compared to previous ones reported on literature in Table 11. The comparison shows the importance of bacterial acclimation process prior to cyanide degradation (KAO et al., 2003; ROSARIO, 2017) and shows the importance of carrying a negative control assay in order to monitor abiotic degradation of cyanide caused by low pH or due to media composition. Furthermore, cyanide degradation could be improved by the addition of nutrient sources as yeast (MEKUTO et al., 2013; NALLAPAN MANIYAM et al., 2013; KARAMBA et al., 2015) in order to promote cell growth.

6 CONCLUSIONS

From the present study, it can be concluded that:

- Cassava-processing wastewaters content free-cyanide and nutrients in concentrations that allow the adaption of cyanide-resistant microorganisms.
- Sixteen cyanide-resistant strains were isolated from the cassava-processing industry effluent. Within the isolated strains, seven Gram-positive rod-shape bacteria and nine Gram-negative rod-shaped bacteria were isolated belonging to the *Corynebacterium glutamicum*, *Klebsiella oxytoca*, *Serratia marcescens*, *Enterobacter asburiae*, *Enterobacter kobei*, *Enterobacter cloacae*, *Bacillus pumilus*, and *Cellulosimicrobium cellulans* species.
- From the 16 isolated organisms, *Corynebacterium glutamicum*, *Klebsiella oxytoca*, *Serratia marcescens*, *and Bacillus pumilus*, strains showed the greatest cyanide degradation potential attenuating cyanide concentration by 28.2%, 25%, 19.5%, and 36.6%, respectively after a 16-hours incubation at 185RPM 30˚C and pH8. The negative control showed a degradation rate below 5% which indicates that the decrease in cyanide concentration was due to microbial activity. Therefore, these strains can be potentially used in the biotreatment of free-cyanide.
- *Corynebacterium glutamicum*, *Klebsiella oxytoca*, *Serratia marcescens*, and *Bacillus pumilus* strains were assessed in their susceptibility to pH and cyanide initial concentration. *Klebsiella oxytoca* and *Bacillus pumilus* strains tolerated pH until 9. *Serratia marcescens* strain decreased its degradation rate at pH above 8. Above pH10, cyanide biodegradation is less effective and at pH11 is almost impossible. Cyanide degradation in alkaline environments would need a prior adaptation of strains to treat solutions with pH above 10.
- *Corynebacterium glutamicum*, *Klebsiella oxytoca*, *Serratia marcescens*, and *Bacillus pumilus* strains were tested to degrade

cyanide at pH8 185RPM 30˚C with 150, 300, 500 and 1000mg.L^{-1} of free-cyanide initial concentration. Strains kept their cyanide degradation ability even in concentrations of 1000mg.L^{-1}. However, degradation rates were lower when the initial cyanide concentration was higher.

- Isolated *Klebsiella oxytoca* and *Serratia marcescens* strains were selected for more detailed studies. Bacterial growth analysis showed that both strains achieved half of their exponential growth phase when cultivated in LB media after 3h of incubation with an optical density of 0.8 and a culture concentration of 10^{10}CFU.mL^{-1}.
- Degradation rates for the isolated *Klebsiella oxytoca* and *Serratia marcescens* strains was calculated to be 2.68mg.L^{-1}.h^{-1} and 2.15mg.L^{-1}.h^{-1}, respectively when using cyanide as a sole nitrogen source, sodium acetate as a carbon source, pH8 and incubated at 30°C and 185RPM.
- Using sodium acetate as a carbon source resulted in higher cyanide degradation by isolated *Klebsiella oxytoca* and *Serratia marcescens* strains, compared to starch and glycerol. Strains achieved 27 and 23% of cyanide removal, respectively, after 16h incubation.
- The optimal incubation conditions for cyanide degradation by isolated *Klebsiella oxytoca* and *Serratia marcescens* strains were both at 30°C. For *Klebsiella oxytoca* strain, rotation speed during incubation did not show an effect on cyanide removal; whereas for *Serratia marcescens* strain, a higher cyanide degradation was achieved when incubated at 235RPM.
- Under optimal conditions, isolated *Klebsiella oxytoca* and *Serratia marcescens* strains were able to degrade cyanide by 43 and 41%, respectively after 24hours of incubation, showing the potential use of native bacteria in the treatment of cyanide-containing effluents.

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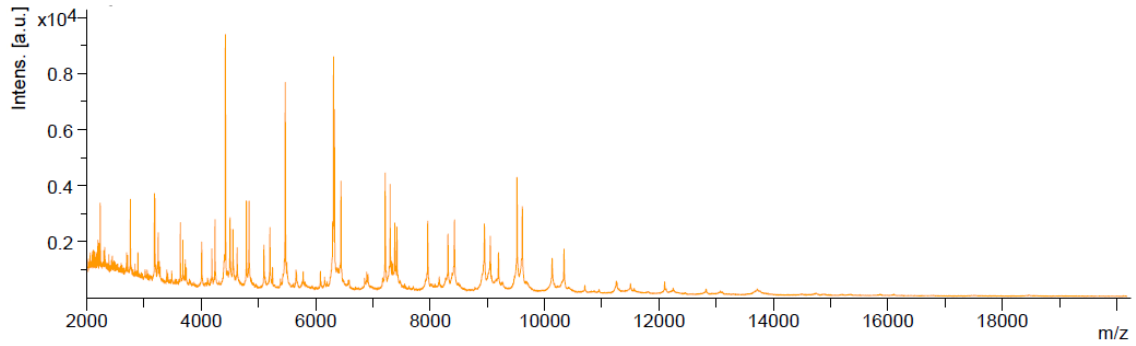
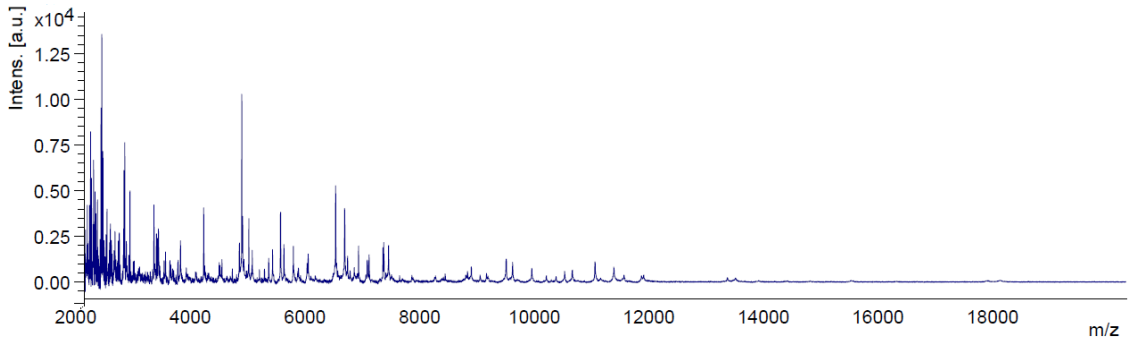
ANNEX A – Protein spectra of isolated strains

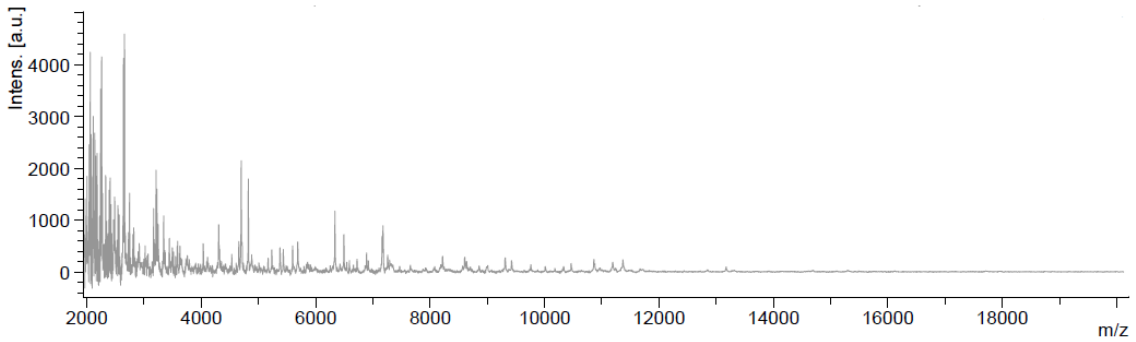
N1 Strain *Klebsiella oxytoca* Score:2.298



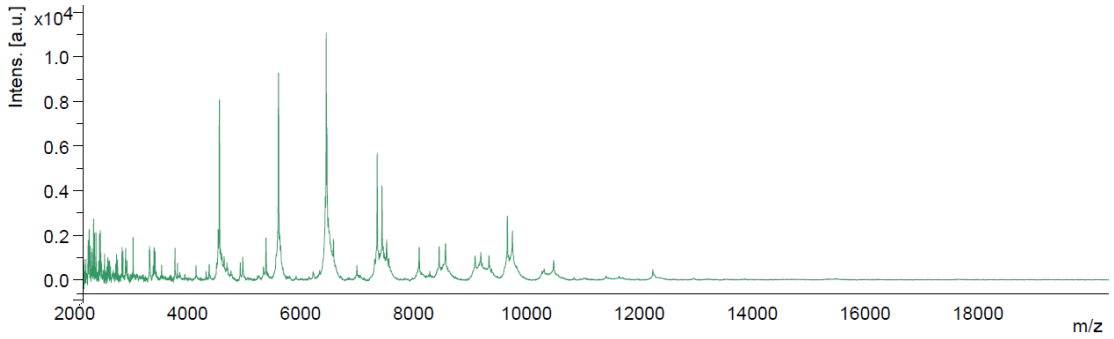
N2 Strain *Corynebacterium glutamicum* Score:2.135



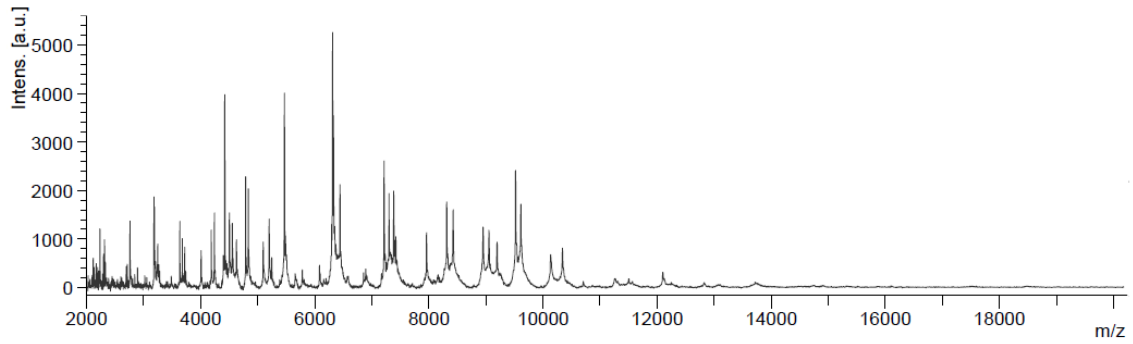
N3 Strain *Corynebacterium glutamicum* Score:1.936



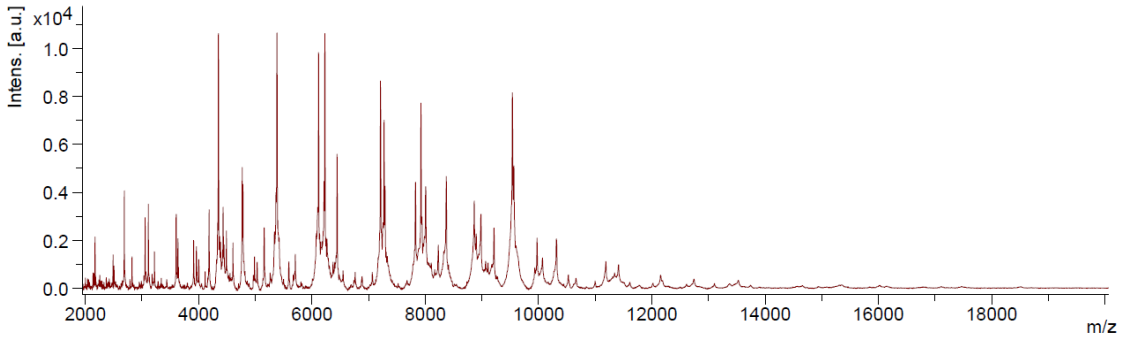
N4 Strain *Klebsiella oxytoca* Score:2.288



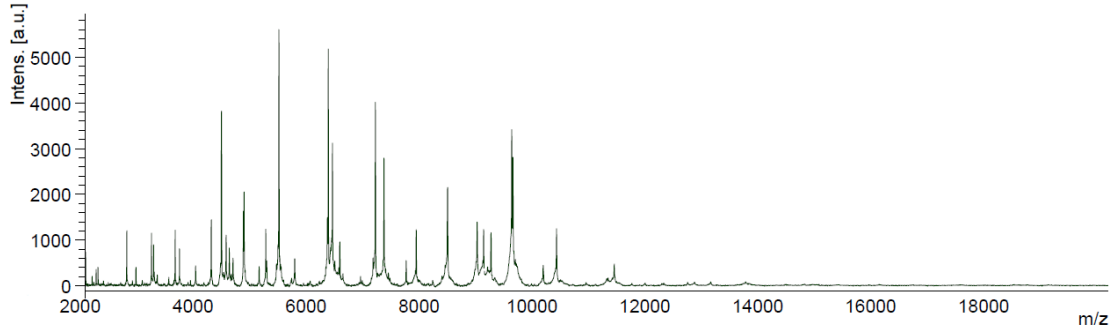
N5 Strain *Klebsiella oxytoca* Score:2.494



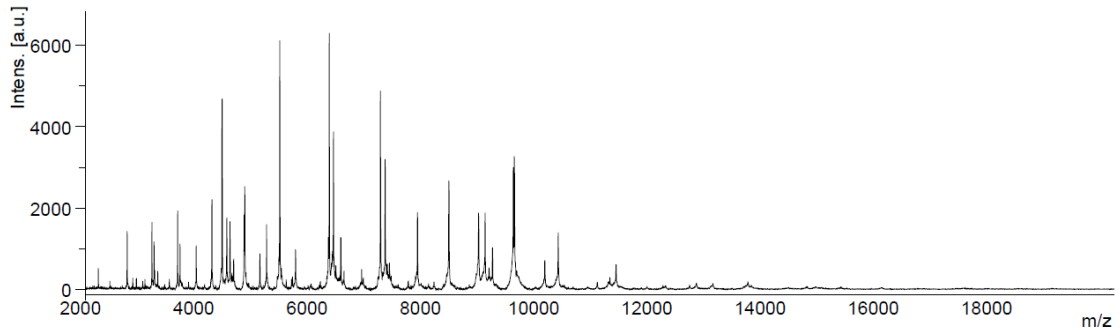
N6 Strain *Serratia marcescens* Score:2.377



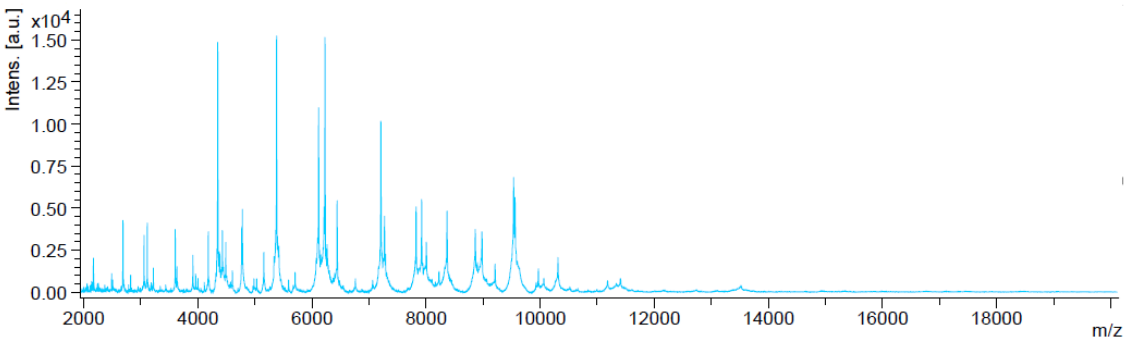
N7 Strain *Enterobacter asburiae* Score:2.338



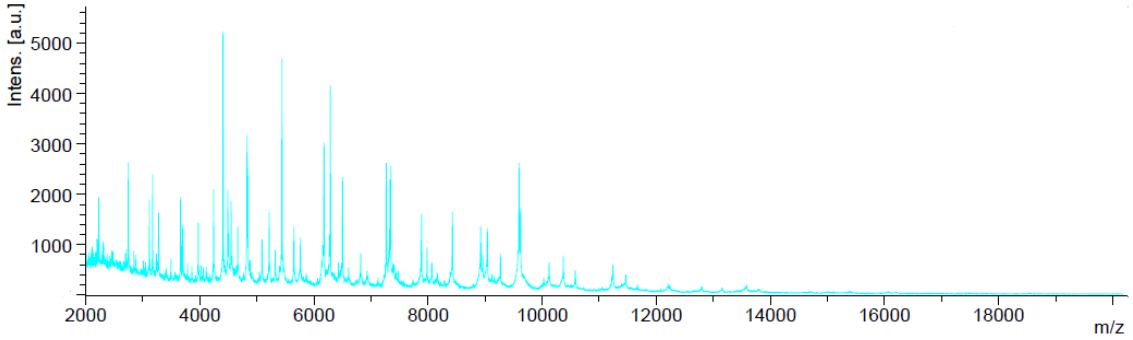
N8 Strain *Enterobacter kobei* Score:2.27



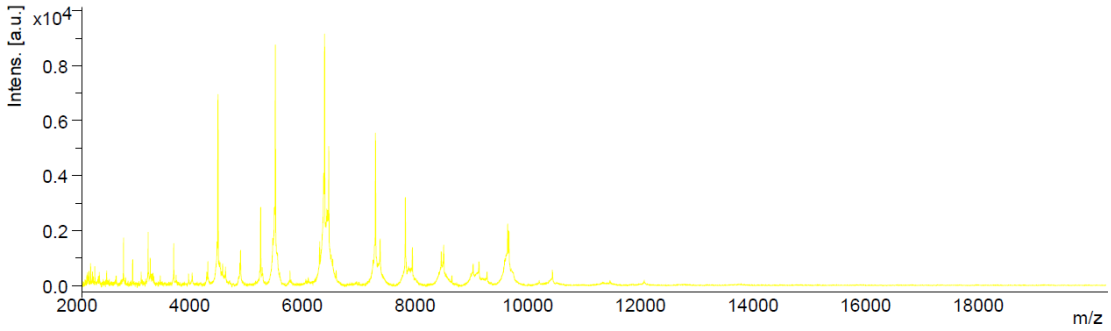
N9 Strain *Serratia marcescens* Score:2.379



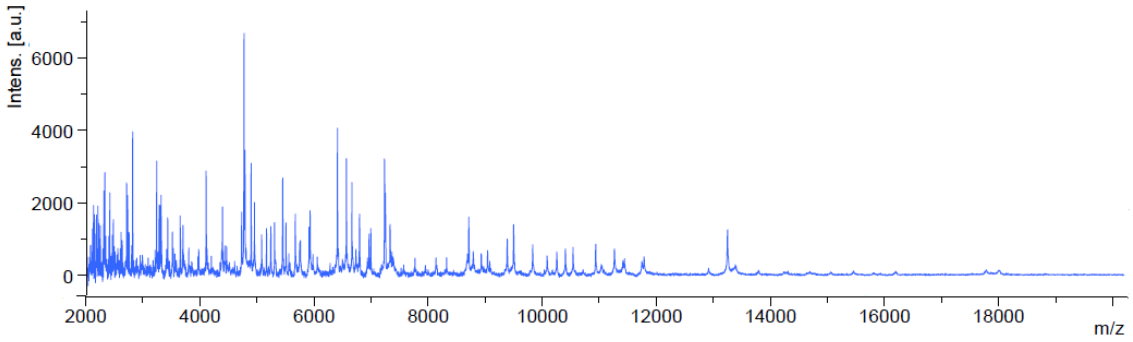
N10 Strain *Serratia marcescens* Score:2.305



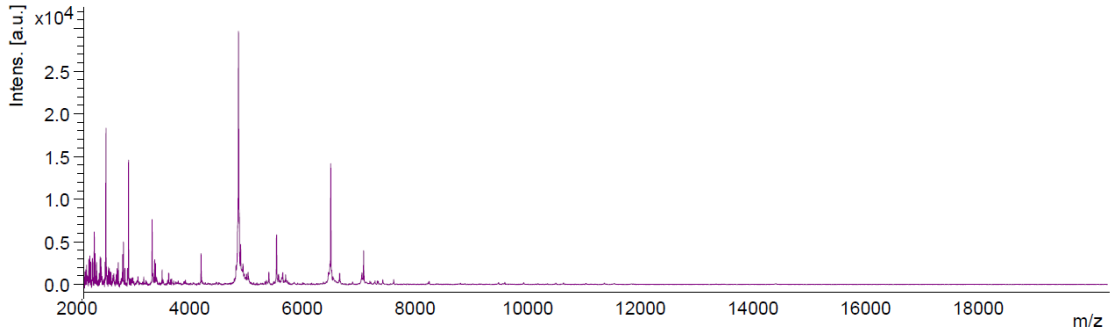
N11 Strain *Enterobacter cloacae* Score:2.265



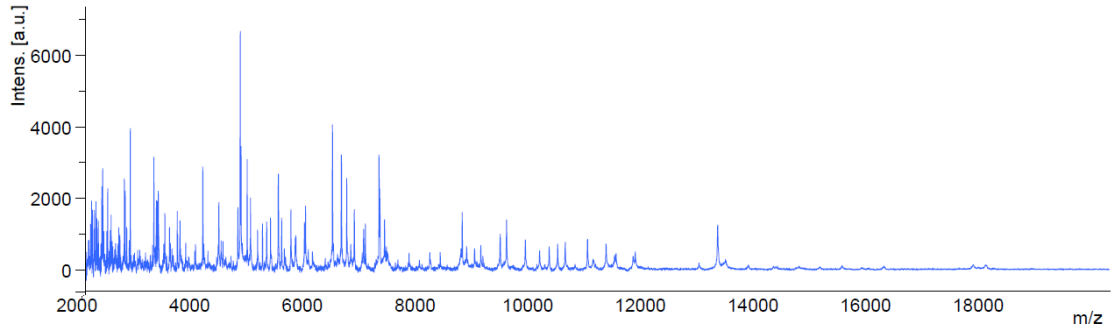
N12 Strain *Corynebacterium glutamicum* Score:2.12



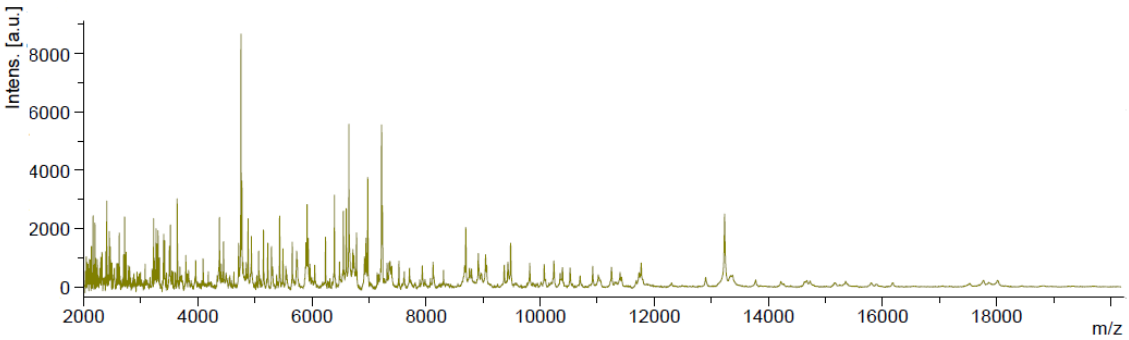
N13 Strain *Bacillus pumilus* Score:2.012



N14 Strain *Cellulosimicrobium cellulans* Score:1.897



N15 Strain *Corynebacterium glutamicum* Score:2.052



N16 Strain *Corynebacterium glutamicum* Score:1.782