Morphological and physiological characterization and biomass production of *Burkholderia pyrrocinia* (BRM 32113)

Caracterização morfológica e fisiológica e produção de biomassa de Burkholderia pyrrocinia (BRM 32113)

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ABSTRACT

We aimed to evaluate the viability of agroindustry residues for *Burkholderia pyrrocinia* (BRM32113) biomass production. BRM32113 was morphological and physiologically characterized; carbon: nitrogen ratio, optimal pH and osmotic conditions were determined and carbon sources profile analyzed. Biomass production was tested with glycerol, molasses, and rice brain, and quantification was obtained by counting colony-forming

unity (CFU). BRM32113 cells are bacilli shaped, and the colony is dark pink gram-negative with high capacity to assimilate carbon sources, producing 8.9E+10 CFU.mL^{-1} in glycerol-containing medium. Optimal C:N was from 2.5 to 3.5, pH ranged from 8.0 to 9.5, and the maximum growth was obtained in period from 6 to 8 hours. The growth rate was 0.332 h^{-1}, and the optimal NaCl concentration was 1 and 2%. Crude glycerol as carbon source was superior. We proved that it is viable to produce biomass from agroindustry waste.

Keywords: agribusiness, bacteria, bioproduct, prospecting, waste.

RESUMO

O nosso objectivo era avaliar a viabilidade dos resíduos da agro-indústria para a produção de biomassa da pirrocinia do Burkholderia (BRM32113). A BRM32113 foi morfológica e fisiologicamente caracterizada; a razão carbono: azoto, pH óptimo e condições osmóticas foram determinadas e o perfil das fontes de carbono analisado. A produção de biomassa foi testada com glicerol, melaço e cérebro de arroz, e a quantificação foi obtida através da contagem da unidade formadora de colónias (UFC). As células BRM32113 têm forma de bacilos, e a colónia é gram-negativa rosa escuro com alta capacidade de assimilar fontes de carbono, produzindo 8,9E+10 CFU.mL-1 em meio contendo glicerol. O C:N óptimo foi de 2,5 a 3,5, o pH variou de 8,0 a 9,5, e o crescimento máximo foi obtido no período de 6 a 8 horas. A taxa de crescimento foi de 0,332 h-1, e a concentração óptima de NaCl foi de 1 e 2%. O glicerol bruto como fonte de carbono era superior. Provámos que é viável produzir biomassa a partir de resíduos da agro-indústria.

Palavras-chave: agronegócio, bactérias, bioproduto, prospecção, resíduos.

1 INTRODUCTION

Interest in microorganisms for biological control in agriculture has grown in recent years, but information on the biomass production processes and formulations of these microorganisms is still scarce. In general, formulations may be in powder or liquid form. The liquid formulation is composed of bioagent biomass in emulsions containing the ingredients that can guarantee the stability and effectiveness of the product (Schisler, 2004). However, to obtain an effective bioproduct, a microbiological fermentation process of quality is necessary, which guarantees the stability and effectiveness of the microorganisms (Legget et al., 2011). An essential aspect of the fermentation is to find a culture medium that contains nutritional characteristics required by microorganisms, such as carbon, nitrogen, and micronutrients. Together with it, using low-cost raw material with low residue generation and high yield (Tabosa et al., 2009) can maximize the bacterial biomass production

In industry, the most commonly used carbon source is glucose, but renewable raw materials are now being introduced to reduce the costs of the microorganism cultivation process, and to achieved this, tests are conducted with other carbon sources. The use of a renewable carbon source, from industry residues such as glycerol, sugar cane molasses, and rice bran seans to be a viable alternative. It can reduce the process expences, since, from an economic point of view, expences with carbon sources contribute significantly to the final cost of the process (Kitamura, 2013; Tabosa et al., 2009).

Glycerol is the common name for the organic compound 1,2,3-propanetriol (OH-CH 2 -CH (OH) -CH 2 -OH). For being a byproduct of biodiesel production, it had an increasing rise in its availability (Beatriz et al., 2011) and its exploitation in industrial use can make biodiesel production competitive in the fuel market, reducing the production costs. There are several alternatives for its use, among them the use in animal feed (Apolinário et al., 2012) and in bacterial fermentations to obtain biomass for bioproducts formulation.

Sugarcane molasses are a high abundance of residue in Brazil, the largest producer of sugarcane (CONAB, 2018), and also the first in the production of sugar and ethanol in the world (MAPA, 2015). It is also cheaper than glucose and contains trace elements and various vitamins that can be used as a microbiological growth enhancer (Rodrigues, 2005). Rice bran stands out as an essential byproduct of rice processing. It accounts for about 5 to 8% of total rice grains and represents one of the most nutritious parts of the grain. It is composed of protein, dietary fiber, and functional compounds such as oryzanol and tocotrienols, as well as lipids (Chaud et al., 2009; Walter et al., 2008). Its composition is rich in nutrients compared to other byproducts of rice processing (Chaud et al., 2009).

The first species of the Burkholderia, *B. cepacia*genus was first described by Burkholder in 1950, and was known as a single species until it was classified as a complex of ten narrowly related genomic species, subclassed into genomovars (Mahenthiralingam & Vandame, 2005; Ren et al., 2011; Yabuuchi et al., 1992). *Burkholderia pyrrocinia* belongs to the *Burkholderia cepacia* (Bcc) complex. Bacteria of this genus are often found in the rhizosphere of different plant species, can produce antifungal compounds, and protect plants against disease through root colonization (Mahenthiralingam & Vandame, 2005). In contrast, some Bccs are pathogenic to humans, responsible for

causing infection in patients with cystic fibrosis (Chiarini et al., 2006) and pathogenic to plants, such as *B. cepacia* that causes rot in onion bulbs [6].

Mahenthiralingam & Vandame (2005), demonstrated that Bcc has ten genomic species called genomovars, which differ among themselves in genetic characteristics and resemble phenotypic characteristics. Some studies have been carried out to differentiate Bcc isolates into biological and clinical agents. Lee et al. (2013) and Ren et al. (2011) sequenced the 16SrRNA region and the recA gene from two *isolates B. pyrrocinia* from the rhizosphere. The authors concluded that both B. pyrrocinia´s isolates belong to genomovar IX, which is known to cluster nonclinical Bcc species (Mahenthiralingam & Vandame, 2005).

The *B. pyrrocinia* strain (BRM 32113) under study is a rhizobacterium isolated from the highland rice rhizoplane (Filippi et al., 2011). Previous works have already proved its efficacy in promoting growth and control of blast in rice(Filippi et al., 2011; Côrtes et al., 2015; Nascente et al., 2017; Sousa et al., 2017). Despite a considerable amount of data on the efficiency of biological agents in the control of rice diseases, there is still no registered biological product for rice cultivation in Brazil (AGROFIT, 2018). Therefore, knowing the morphological and physiological characteristics of this bioagent may favor the optimization of the production processes of another component for integrated blast handling. Therefore, the objective of the present work was to perform the morphological and physiological identification and to produce biomass of *B. pyrrocinia* rhizobacteria (BRM 32113) utilizing agro-industrial residues.

2 MATERIAL AND METHODS

2.1 MICROORGANISMS

The *B. pyrrocinia* BRM 32113 bacteria belong to the Embrapa Microbial Collection and preserved by the Castellani (bacteria water suspension stored at 8°C).

2.2 MORPHOLOGICAL CHARACTERIZATION

2.2.1 Scanning Electron Microscopy (SEM)

Previously grown bacterial colonies were transferred to test tubes containing 5 mL of 0.85% sterile saline solution followed by vortex homogenization. Fifty µL of bacterial suspension was deposited on a dry and clean coverslip, and the sample was fixed for 30

minutes with 100 µl of 3% glutaraldehyde (diluted in 0.1 M sodium phosphate buffer, pH 7.2). After fixation, washing was carried out three times with sodium phosphate buffer, five minutes for each wash. Followed by serial alcoholic dehydration of 30%, 50%, 70%, 80%, 90%, and 100%for five minutes each series.

At the end of dehydration, the coverslip was covered with HDMS (hexamethyldisilazane) for six minutes and dried for 20 minutes at room temperature. Alcohol at100% (P.A.) was used for drying, which was carried out under the critical condition of the carbon dioxide (CO2), made in the Autosamdri® critical point apparatus, 815, Series A. The samples were arranged in stubs for metallization in the Denton Metallizer Vacuum, Desk V., where they were covered by gold for posterior analysis in MEV model Jeol, JSM - 6610, equipped with EDS, Thermo scientific NSS in the Multiuse Microscopic Laboratory (LabMic) of UFG.

2.2.2 Gram staining

In a clean glass slide, a smear from pure bacterial colonies was prepared with one drop of sterile distilled water. To fix the smear, the glass slide was quickly dried (the side opposite the smear)for five consecutive times using a fire flame. The glass slide with the dried smear was dyed with violet crystal and Lugol solution for one-minute, absolute alcohol for fifteen seconds, and gram fuchsin for 30 seconds. Next, the glass slide was rapidly washed with distilled water and dried at room temperature. Lastly, a mineral oil drop was added for light microscope observation and staining identification.

2.2.3 Metabolic diversity of *B. pyrrocinia* by analyzing the profile of the different carbon sources used

The assimilation profile of carbon sources was evaluated in an Eco plate Biolog microplate (Biolog, Inc., Hayward, CA). The microplate was composed of 96 wells containing 31 different carbon sources plus one blank well, in triplicate, grouped into six classes of nutrients: amino acids, amines, carbohydrates, carboxylic acids, polymers, and miscellaneous (Fig. 1).

The *B. pyrrocinia* was grown in nutrient broth culture medium and maintained under constant stirring at 150 rpm for 48 h at 28 ° C. The suspension concentration was then adjusted to 10^5 CFU.mL^{-1} in a spectrophotometer (Hach DR2800) at a 620 nm

wavelength. Next, 100 µl of bacterial suspension was transferred to each Eco plate Biolog well, which was incubated in the dark at 25 ° C for 96 hours under constant stirring at 150 rpm at 28 ° C.Cellular growth was evaluated at 0, 24, 48, 72 and 360 hours in a microplate reader (Epoch ™ - Biotek) with the aid of the Gen5 software (Biotek, Vermont, USA) at 590 nm. Carbon sources (AMR) metabolization was calculated by the difference between the optical density of the wells containing carbon sources and the control (white).

2.3 PHYSIOLOGICAL CHARACTERIZATION

2.3.1 Optimum carbon and nitrogen (C: N) ratio for bacterial biomass production.

Tests were performed with three independent variables (C: N, pH, and time), performing a complete factorial design (2^2), with five central points, using an experimental design of the central rotational compound (CCRD). The assay was performed in two steps. The first one consisted of an exploratory assay 1, to identify the C: N ranges and time in which they should be analyzed more accurately. The second step consisted of two more assays, 2 and 3, to identify the best C: N ratio, pH, and time. The tests were performed randomly, and data were statistically analyzed using the Action® software, with a 95% confidence level, and response surface curves were obtained.

The minimal media used in these experiments ($(NH_4)Cl$ 0.5g; K_2HPO_4 0.1g; NaCl 0.5g; $MgSO_4.7H_2O$ 0.02g; Water (qsp) 100mL, Glucose and pH variables according to Table 1) had only glucose as a carbon source and ammonium chloride as a nitrogen source. The carbon source amount, and consequently, the C: N ratio, are presented in Table 1 (assays 1, 2, and 3). The incubation temperature was kept constant at 28 °C with stirring at 150 rpm. Biomass production quantification was performed by the serial dilution method and counting of $CFU.mL^{-1}$ in Petri plates containing nutrient agar for test 1. For tests 2 and 3, the biomass quantification was performed by a spectrophotometer at a 620 nm wavelength.

2.3.2 Determination of bacterial growth rate

The growth rate of the bacterial isolate was determined by the angular coefficient of the graph relating to the cell growth (optical density in $\lambda = 620$ nm) and the incubation time (hours). The determination of growth rate was composed of 3 steps: 1) the isolate was grown in 250 mL Erlemeyers containing 50 mL of nutrient broth culture medium and

maintained under constant stirring at 150 rpm for 48 h at 28 °C. Next, 1 ml of the bacterial suspension was transferred to the Erlenmeyer containing 50 ml of the optimum defined growth medium described in Table 1, with the C: N ratio of 3: 1 and pH 8. The flasks were incubated under constant stirring at 150 rpm at 28 ° C. This step was conducted in triplicate. After 24 hours of incubation, the optical density (620 nm) was recorded every hour, over 12 hours. The equation used to determine the bacterial growth rate "k" was: $k = Ln(A/Ao).t\text{-}1$, where Ao corresponds to absorbance value at time zero, A is the absorbance value of bacterial culture in the time t.

2.3.3 Bacterial growth rate under different osmotic conditions

The bacterial isolate was previously grown in culture broth for 48 h at 28 °C. The suspension concentration was then adjusted to 10^5 CFU.mL^{-1} in the spectrophotometer (Hach DR2800) at 620 nm wavelength. Subsequently, 10 µl of suspension was transferred to the wells of the microplate using a multichannel pipette. Each well contained saline solution (140 µl) at the following NaCl concentrations: 0.5; 1; 2; 3; 4; 5; 6; 7; 8; 9 and 10% (triplicate). During the period in which the evaluations were performed, the plate was maintained under stirring (7000 rpm) at room temperature. Optical density (620 nm) was recorded every hour for a 14-hour incubation period in a microplate reader (Epoch ™ - Biotek) with the aid of the Gen5 software (Biotek, Vermont, USA). The equation used to determine the bacterial growth rate "k" was: k = Ln (A / Ao) .t-1, Where Ao corresponds to the absorbance value at time zero, A is the absorbance value of the bacterial culture in the time t.

2.3.4 Use of alternative carbon sources in biomass production

The substrates sugarcane molasses, rice bran, and glycerol were tested as alternative carbon and nitrogen sources in bacterial biomass production with the optimum C: N ratio previously defined, replacing the glucose and ammonium chloride used in the previous test. Incubation temperature was constant at 28°C and stirring at 150 rpm. The evaluations were performed at 24, 48, and 72-hours after incubation, and the biomass production was quantified by the serial dilution method followed by counting of CFU.mL^{-1} in Petri dishes containing nutrient agar medium. As a first step of the present assay, an initial volume of bacterial cells was required for the production of biomass. For the initial

inoculum, *B. pyrrocinia* BRM 32113 was cultivated in the nutrient broth culture medium, incubated in a shaker at a temperature of 28 °C at 150 rpm. After 24 hours of incubation, 1 ml of initial inoculum was transferred to each treatment.

The different carbon doses and sources used for the preparation of the culture media are described in Table2. The culture medium with the best C: N ratio was used as a control. Treatments with glycerol substrate were supplemented with salts and ammonium chloride because of both forms, purified and residue, present only carbon sources for energy supply. Also, for rice bran and molasses, both presented reduced sugars and rich mineral composition, so they were evaluated only with and without the addition of ammonium chloride.

The number of colony-forming units per treatment and per incubation interval was transformed into a decimal logarithm. For the variance analysis and comparison between means by the Tukey´s test ($p < 0.05$) (significance level), the SPSS - statistical analysis software v. 2.1 was used.

3 RESULTS

3.1 MORPHOLOGICAL CHARACTERIZATION

B. pyrrocinia BRM 32113 colonies grown on nutrient presents pink color (Figure 1-A) and pink staining by the Gram's method, presented as gram-negative (Fig 1-B), and the cells are straight bacilli when observed with scanning electron microscopy (SEM) (Fig 1-C and D).

3.2 METABOLIC DIVERSITY OF *B. PYRROCINIA* BY ANALYZING THE PROFILE OF THE DIFFERENT CARBON SOURCES USED

Among 31 treatments (different carbon sources) tested in Eco plate plates, *B. pyrrocinia* BRM 32113 was able to metabolize 32.2% of carbon sources in48 hours, 54% in 72 hours, and a greater number of carbon sources (67%) in 360 hours (Fig.2). The 31 different carbon sources tested in an Eco plate are classified as polymers, carbohydrates, carboxylic acids, amino acids, amines, and phenolic compounds. From 31, 25 different carbon sources were metabolized. The only non-metabolised carbon sources were: D-Xylose, D-Mannitol, α-Cyclodextrin, γ-Hydroxybutyric Acid, Glycogen, D-glucosaminic.

3.3 PHYSIOLOGICAL CHARACTERIZATION

3.3.1 Determination of optimum relation between carbon and nitrogen (C: N) to obtain bacterial biomass

A significant (P=0.05) interaction was observed between the pH and C: N response. Assay 1: curve response shows that the best C: N ratio ranged from 2 to 4, represented by the equation generated by the Action® software: cells concentration (CFU.mL^{-1}) = -3.5x10^{15} + 2.3x10^{15}.CN + 2.2x10^{14}T. -1.6x10^{13} T^2 – 3.8x10^{14} CN^2 with a determination coefficient of R^2= 0.90. The range of optimal C: N ratio is from 2.5 to 3.5, in the range from 6 to 8 hours (Fig. 3, A; B).

Assay 2: regarding the curve response, the pH was significantly influenced by R^2 = 0.96, a positive correlation with pH increase, independent of the C: N ratio. In culture mediums with pH close to 8, cell production approached an absorbance of 1.5, corresponding to a value higher than 1x10^8 CFU.mL^{-1} (Fig. 4; A, B). Assay 3: C: N ratio remained the same, changing only the pH, which ranged from 8.0 to 9.5. The curve response obtained showed a positive correlation between the C: N ratio and pH, with R^2, = 0.71, confirming the previous tests (Fig. 5; A, B).

3.3.2 Determination of bacterial growth rate

Optical density data were transformed to log for linearization of curve growth over time, represented by the equation with R^2= 0.957: k = Ln (A / Ao) .t^{-1}, where: k: microbial growth rate; Ao: absorbance value at time zero; A: absorbance value of the bacterial culture at time t. Linear regression (R^2= 0.957) represents a specific growth rate of *B. pyrrocinia* BRM 32113, in optimum culture medium maintained under constant stirring at 150 rpm and 28ºC, which was 0.332 h^{-1}. It is also considered that the adaptation phase was 0 to 4 hours after inoculation in medium and the exponential phase until the 9-hour period, where the stationary phase began (Fig 6).

3.3.3 Bacterial growth rate under different osmotic conditions

B. pyrrocinia BRM 32113 growth curves were obtained when submitted under different osmotic conditions, established by different NaCl concentrations, expressed by an absorbance measurement at 620nm. Bacteria cells were in the adaptation phase for a3

hour period, then the treatment with 1% NaCl stood out significantly. At 8 hours, there was intense growth at concentrations of 1 and 2% NaCl. The growth rate was moderate at 0.5, 3, and 4% NaCl concentrations and was low in the other concentrations when compared to 1 and 2% concentrations 8 hours after incubation. The stationary phase was started in all treatments after 10 hours of incubation (Fig. 7).

3.3.4 Use of alternative carbon sources in biomass production

B. pyrrocinia BRM 32113 presented the ability to grow in the different carbon sources tested (glycerol, rice bran, and sugar cane molasses), showing no significant differences in growth from the optimal medium containing glucose (1) (Table 3). It was observed that inthe 24 hours, there was similar cell production in all tested culture media enriched with different residues. After 48 hours, there was a decrease in cell production, except for treatments 6 and 7 (molasses and rice bran with nitrogen addition), which showed growth averages similar to optimal medium. At the 72 hours incubation period, it was observed that there was an increase in the production of cells in treatments 3, 4, 5, 6, and 7 contained glycerol, molasses, and molasses with the addition of nitrogen, respectively.

4 DISCUSSION

B. pyrrocinia BRM32113 presented the following morphological features: dark pink, opaque and sticky consistency colonies, circular shape and regular margins, convex elevation, and bright surface (Fig. 1, A), Gram staining showed pink staining, being characterized as gram-negative (Fig. 1B). The shape of cells in SEM was identified as straight bacilli (Fig. 1-C and D). According to Holmes et al. (1998), these are the general characteristics of the *Burkholderia* genus, belonging to the phylum Proteobacteria and the class of Betaproteobacteria.

Determining the profile and metabolic diversity of *B. pyrrocinia* BRM 32113 (Fig. 2), we found that this bacterium presented the high capacity to assimilate carbon sources present in the Eco plate, which is a typical situation found in soils. Within 72 hours, this bacterium had already metabolized 54% of the carbon sources. BRM32113´s origin can explain these results. It was isolated from the rice´s rhizosphere, thus showing a better adaptation to these conditions, and explaining the results obtained by Filippi et al. (2011),

who applied BRM 32113 directly in the soil, and observed efficiency in plant growth promotion and reduction of up to 90% in leaf blast severity. Similar results were also found by Côrtes et al. (2015); Sousa et al. (2017); Nascente et al. (2017) in promoting rice growth and in the same pathosystem.

*B. pyrrocinia´*s (isolate BRM 32113) morpho and physiological studies, are essential to allow a broader understanding of its adaptation mechanisms. It may favor its use in a more efficient way, which can allow obtaining more significant control of the production process and, consequently, the quality of biomass. Several aspects need to be analyzed for the prospection of a microorganism, such as the determination of the best C: N ratio and pH, optimum NaCl concentrations, as well as growth rate calculation.

Todar (2012) describes that usually, the C: N ratio ideal for bacterial growth is 4:1, and the elemental composition of the cells reveals the bacteria's nutritional requirements. However, it varies between bacterial species the length of time a cell needs to metabolize nutrients. It also is important the relationship between their components, their origin, and their functions. For *B. pyrrocinia*, BRM 32113, the best C: N ratio found was 2.5: 3.5, metabolized between six and eight hours of growth (Fig. 3; A, B). Although neutral pH is ideal for most of them, each microbial species has an optimum pH for growth. According to Baka et al. (2013), the growth rate of a microorganism is maximum when the pH value is optimal, and much lower when considering stress values. In the present study, we can prove this fact because, *B. pyrrocinia* BRM 32113 developed better at an alkaline pH in the range from 8.0 to 9.5 (Fig. 4 and 5; A, B).

In previous works (Côrtes et al., 2015; Filippi et al., Sousa et al., 2017), *B. pyrrocinia* BRM32113 proved to be efficient in leaf blast control and induction of resistance in rice when cultured in broth nutrient for 48 hours. However, to optimize the process for obtaining this bacterial inoculum, as well as confirming the previous results, we determined the specific bacterial growth rate was 0.332 h^{-1} (Fig. 6), under optimal conditions. The adaptation phase was 4 hours after inoculation in the medium; the exponential phase was up to 9 hours when the stationary phase started.

The pH, besides being an essential factor for biochemical reactions, also determines the distribution and capacity of microorganisms to metabolize carbon sources in the culture medium. The total concentration of inorganic carbon and other culture medium components changes the osmotic pressure of the medium, influencing cellular

physiology (Lima et al., 2007). So, it is necessary to supplement culture medium with salts, such as NaCl, which maintains the osmotic equilibrium of medium as well as serving as a source of Na and Cl ions.

B. pyrrocinia BRM 32113 cultivated under various NaCl concentrations, presented a higher growth rate at 1% and 2% concentrations, with up to 63% growth when compared with other treatments (Fig.7). It presented moderate growth and resistance to saline stresses at concentrations of 0.5%, 3%, and 4% (Fig. 7). According to Luderitz et al. (1982), gram-negative bacteria present as a primary cell wall component, a complex of lipopolysaccharides, significant for partial control of entry and exit of some substances from the cell to medium, or vice versa. Therefore, the composition of its cell wall is a crucial feature linked to its stress resistance that has been submitted. However, for other concentrations, it showed low resistance.

The residues, with their richness in nutrients, mainly as a source of vitamins and minerals, represent a byproduct of Brazilian agroindustry with the potential to be used in the supplementation of microbiological means (Chaude et al., 2009). However, not all microorganisms have the capacity to metabolize these nutrients efficiently. Our results demonstrated that the performance of *B. pyrocinia* BRM 32113 was promising in the utilization of agroindustrial byproduct nutrients.*B. pyrrocinia* BRM 32113 produced high cell concentration in all media supplemented with residues. Although there was no significant difference in cell production between them, it was observed that at the period of 24 hours incubation, glycerol residue was highlighted in cell production when compared to other treatments and glycerol.

Glycerol has free passage through the cell membrane (Champe et al., 2009), and can be easily absorbed by some microorganisms, accelerating metabolism, as demonstrated in the present essay. In the 48 hours, there was a decrease in cell production but remained stable for up to 72 hours. Similar results were found by Tabosa et al. (2009), who evaluated the effect of glycerol with a carbon source in submerged fermentation of the yeast from the Rhodotorula genus, which showed significant growth in the medium containing only glycerol as a carbon source.

At 48h incubation, rice bran favored bacterial cell growth, which developed more with and without the addition of nitrogen when compared to the other substrates (Table 3). Rice bran is composed of hemicellulose, cellulose, starch, β-glycans (Malekian et al.,

2000), and lipid nutrients, corresponding to approximately 20% of weight in oils (AGROSOFT, 2006). Both carbohydrates and oils can limit the use of bran because they are difficult to break down. In that case, the carbon stays trapped in the fibers. This information would explain why the bacterial growth remained stable with no increase or decrease in cell production as nutrients were being released slowly.

At the 72 hours incubation period, medium containing molasses stood out along with rice bran (Table 3). Among all residues, molasses is more abundant in carbon source, approximately 50% (Feltrin et al., 2000). In culture media, without the addition of a nitrogen source, it had the natural C: N ratio of 260:1. Growth delay in culture medium containing molasses can be explained by the time necessary for bacterial cells to adapt to a physiological change due to modifications of nutrient concentrations (Lee et al., 2013). In addition to salt, other compounds can cause cell stress, such as excess sugars. These, at high concentrations in the medium, cause osmotic stress, but lighter than the salts, since they promote a fast balance between intra and extracellular media (Poffo & Silva, 2011).

In studies involving other microorganisms (Cazetta & Celligoi, 2005; Feltrin et al., 2000), conducted with sugarcane molasses, with and without the addition of a nitrogen source, both substrates were efficient in the production of biomass, as observed with *B. pyrrocinia* BRM 32113, which produced biomass both with and without the addition of a nitrogen source in media containing sugar cane molasses and rice bran.

5 CONCLUSION

B. pyrrocinia BRM 32113 is gram-negative, with straight bacilli cells format, and has a high ability to assimilate carbon sources present in the soil. The optimum ratio between C: N and pH for its production are 2.5 to 3.5 and 8.5 to 9.5, respectively. The use of agro-industrial residues in bioprocesses is highly promising, since, in addition to recycling agroindustrial wastes, it also reveals itself as an efficient carbon source in the production of viable bacterial biomass, with optimized and low-cost processes.

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Fig. 1 Morphological characteristics of *Burkholderia pyrrocinia* BRM32113. a Purified colony after 48h growth in nutrient agar medium. b Smear cells of Gram staining, observed under an optical microscope. c Scanning electron microscopy of cells at 5 µm and d Scanning electron microscopy of purified cells at 1 µm.

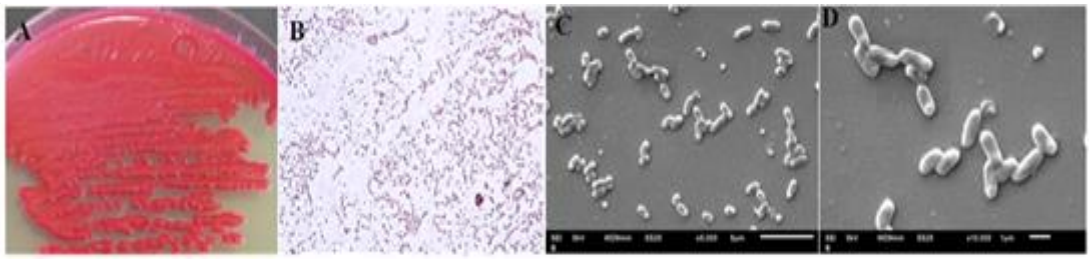

Fig. 2 a Average respiration of C-sources by *B. pyrrocinia* BRM32113 in different hours of incubation. *AMR: average metabolic response. The AMR is calculated as the average of the mean difference between the O.D. of the C-source-containing wells and the control well. b Number of substrates utilized by *B. pyrrocinia* BRM32113. *CMD: community metabolic diversity. CMD is calculated by summing the number of positive responses (purple-colored wells) observed following incubation.

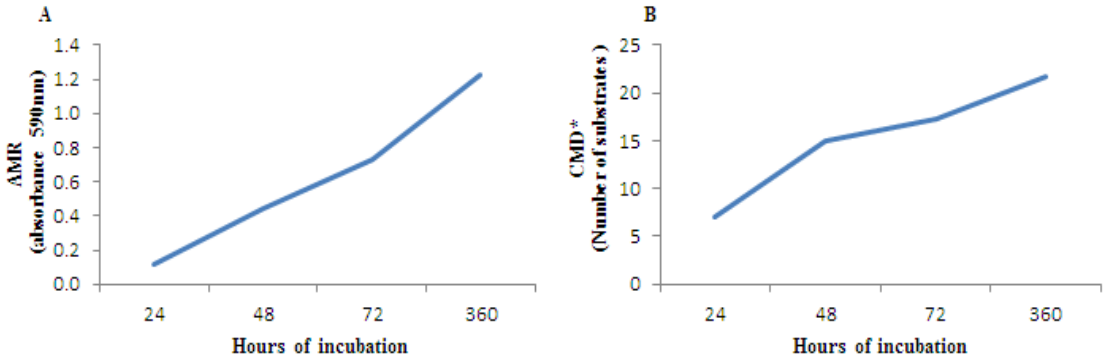

Fig. 3 Contour curve a and surface response curve b of *B. pyrrocinia* BRM32113 by carbon nitrogen reaction (C: N) and time (assay 1)

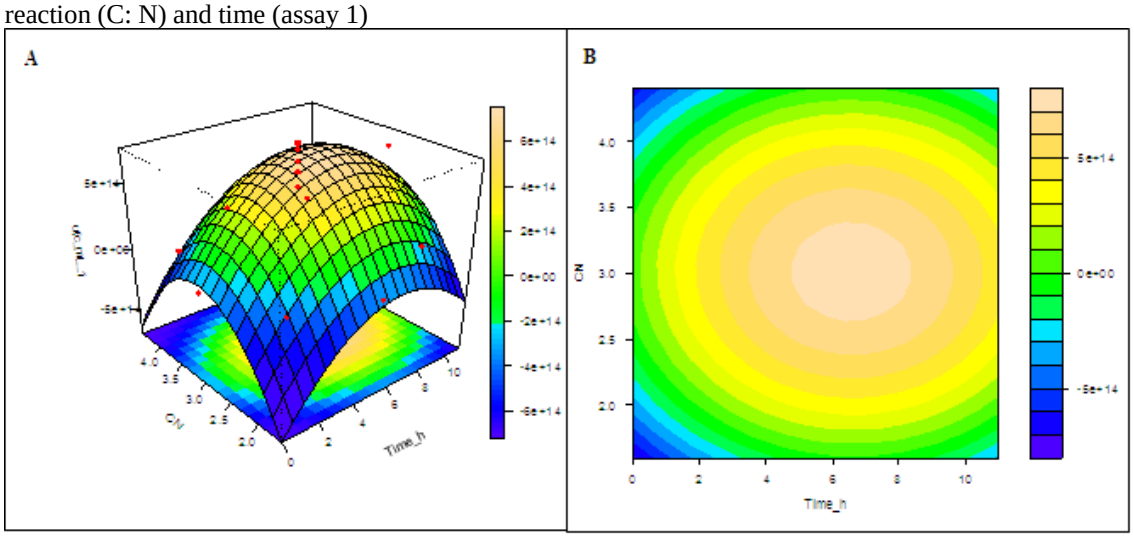

Fig. 4 Contour curve a and surface response curve b of *B. pyrrocinia* BRM32113 by carbon nitrogen reaction (C: N) and pH (assay 2).

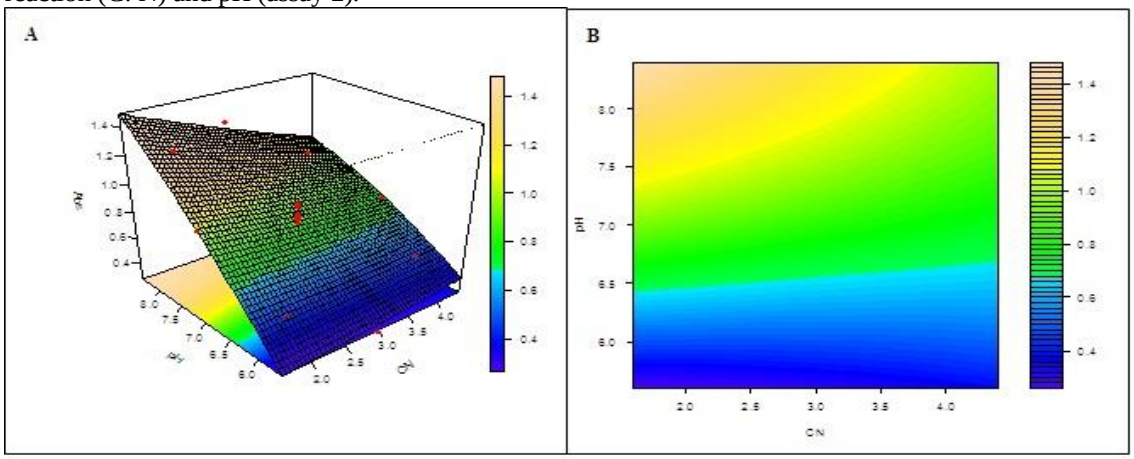

Fig. 5 Contour curve a and surface response curve b of *B. pyrrocinia* BRM32113 by carbon nitrogen reaction (C: N) and pH (assay 3).

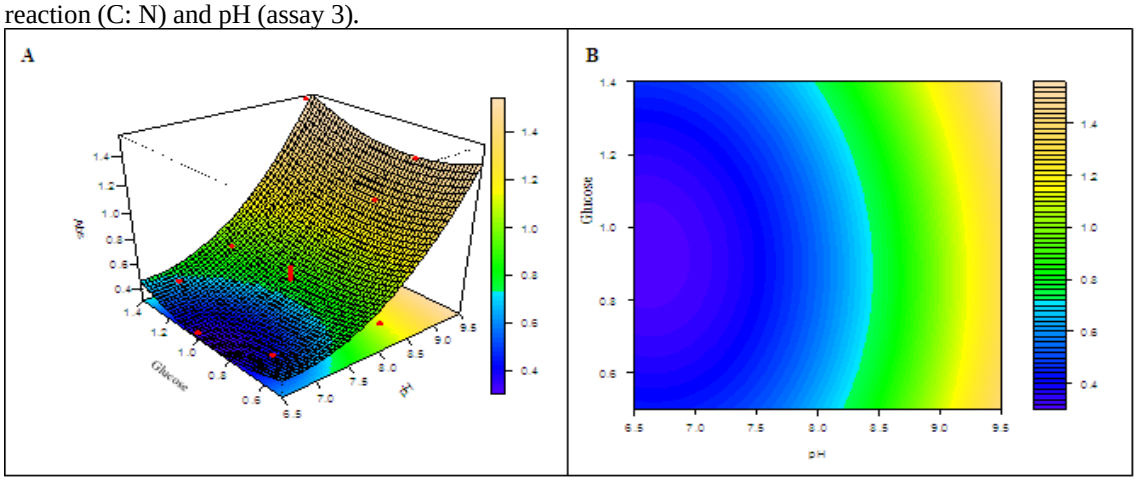

Fig. 6 Growth curve of *B. pyrrocinia* BRM32113 expressed in Ln(A/Ao) by time in optimal culture medium. *Ln: neperian logarithm; Ao: absorbance value at time zero, A: absorbance value of bacterial culture at time t.

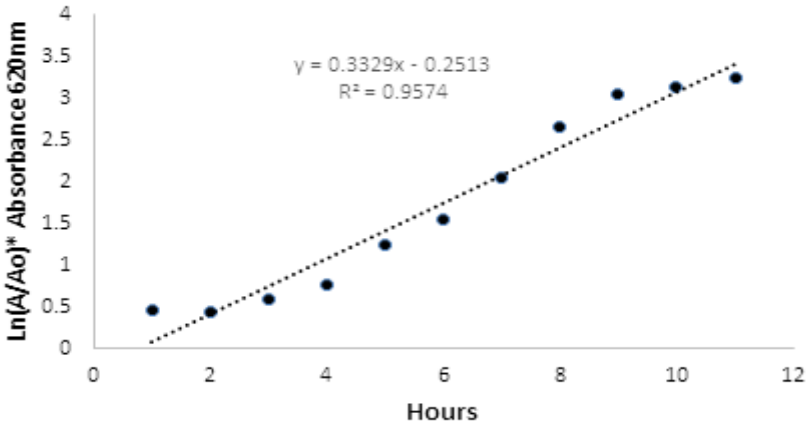

Fig. 7 Growth curve of *B. pyrrocinia* BRM32113 expressed in Ln(A/Ao) by time under different osmotic conditions. *Ln: neperian logarithm; Aa: absorbance value at time zero, A: absorbance value of bacterial culture at time t.

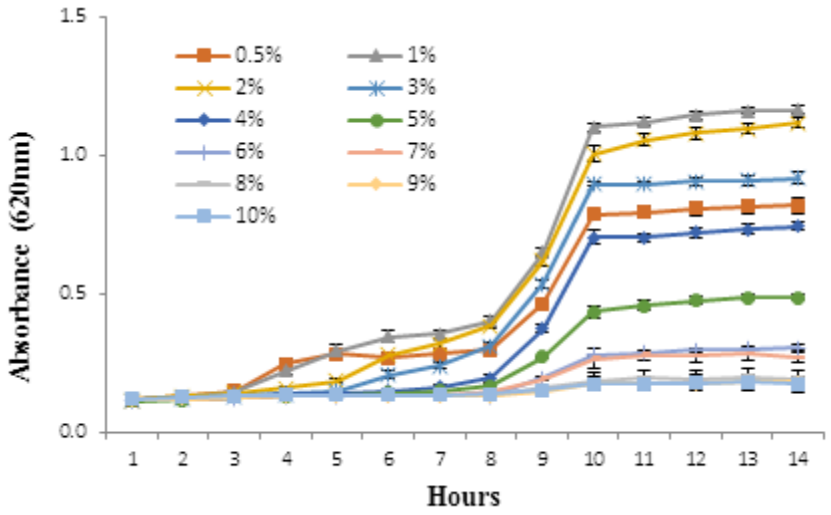

Table 1. Complete factorial experimental design 2^2 variables and their respective real and coded levels in *B. pyrrocinia* BRM 32113 production of biomass of tests 1, 2 and 3.

C:N relation	C:N (m/m) or (g/g)	Glucose (g)	Assay 1			Assay 2	Assay 3
			Time	Hours	pH encoded	pH	pH
-1,41	1,6:1	0,5	-1.41	0	-1,41	5,6	6,5
-1	02:01	0,7	-1	1.6	-1	6	6,9
0	03:01	1,0	0	5.5	0	7	8
1	04:01	1,3	1	9.4	1	8	9,1
1,41	4,4:1	1,4	1.41	11	1,41	8,4	9,5

Table 2. Dosages of different carbon sources used in culture media for production of *B. pyrrocinia* BRM 32113 cells

	Treatments	**% C (g/g)**	**C (% p/v)**	**C:N relation**	**Quantity (g)**
1	Glucose (Control)	40	0.39	03:01	0,98
2	Glycerol PA	39	0.39	03:01	1
3	Glycerol residue	39	0.39	03:01	1
4	Molasses	20	0.39	39:01	1.95
5	Molasses with nitrogen (N)	20	0.39	03:01	1.95
6	Rice bran	27.2	0.39	13:01	1.43
7	Rice bran with N	27.2	0.39	03:01	1.43

Table 3. Production of *B. pyrrocinia* BRM 32113 cells at different times in the culture media with agroindustrial residues as the main source of carbon

Treatment/ Time	24h	48h	72h
1 (glucose)	2.8E+09 Aa	1.6E+09 Ab	8.9E+08 Bb
2 (glycerol PA)	5.7E+09 Aa	2.3E+08 Ca	1.0E+08 Ba
3 (glycerol residue)	8.9E+10 Aa	2.7E+08 Ca	6.7+08 Ba
4 (molasses)	4.4E+09 Aa	5.5E+08 Bca	1.0E+09 Ba
5 (molasses with N)	7.6E+08 Aab	2.1E+08 Cb	9.7E+08 Ba
6 (rice bran)	2.2E+09 Aa	1.2E+09 Aba	5.1E+09 Aba
7 (rice bran with N)	6.1E+09 Aab	1.5E+09 Ab	8.5E+09 Aa

Different letters represent significant difference ($p \leq 0.05$) according to Tukey's test. Capital letters define effect of treatments, and lowercase letters define the effect of time.