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# OBTAINING AND EVALUATING GROWTH PARAMETERS OF SPORE *BACILLUS ATROPHAEUS* ATCC 9372 USING ORANGE WASTE AS A SUSTAINABLE CROP MEDIUM

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**Abstract:** Brazil is the world's largest producer of orange juice, generating a large amount of waste that could be used as a substrate by microorganisms, adding value to what would otherwise be discarded. *Bacillus atrophaeus*, they are bacteria of great interest in the pharmaceutical, hospital and industrial areas. The use of waste from orange juice processing was evaluated as an alternative culture medium to obtain orange spores. *B. atrophaeus*, 100 mL of water was added to the orange pomace (from 1.0 g to 20.0 g) and sterilized at 121 ºC. Tax rate 0,1 $g.L^{-1}$ of spores of *B. atrophaeus* was inoculated in bagasse medium and incubated at 37ºC for up to 6 days. The maximum growth and productivity obtained were $\mu_{max} = 0.0238\ h^{-1}$ and $P_x = 0.0787$ $g.\ L^{-1}.h^{-1}$, respectively for 5.0 g of bagasse. The values of biomass, pH, spores and thermal resistance at 102ºC were determined. An increase in pH was observed after cultivation and the spore concentration reached $1,73 \times 10^9$ spores. $mL^{-1}$ and $5,75 \times 10^9 spores.mL^{-1}$ after 3 and 6 days of incubation, respectively. The decimal reduction times determined varied from $D_{102C} = 0,92$ min at $D_{102C} = 2,71$ min. and $D_{102C} = 1,34$ min at $D_{102C} = 3,98$ min after 3 and 6 days of incubation, respectively. All media containing orange juice residues allowed the development of spores, with adequate thermal resistance, showing that these can be applied to obtain products of commercial importance.
**Keywords:** *Bacillus atrophaeus*, biological indicator, agro-industrial residues, orange pomace.

## INTRODUCTION

Orange juice is an important trade in the world. Brazil is the largest producer, corresponding to three-quarters of exports (1.1 million tons), followed by Mexico (178 tons) and the United States (85 tons) estimated for 2022/23 (USDA, 2023). Fresh fruit is submitted to processing in orange juice production, which wastes a considerable number of residues because they do not have application in agro-industrial processes. Nearly half of one orange weight is given to juice, and the other half is treated as residue, also known as bagasse, containing peel, internal tissues, pulp, and seeds, that is rich in nutrients and may be used as the substrate for microbial cultivation and production of by-products with industrial interests, increasing their value (MARTINEZ-TRUJILLO et al., 2011; MOHSIN et al, 2022).

The orange bagasse has many nutrients in its composition; the organic fraction includes soluble sugars (21.06%), pectin (18.45%), proteins (19.78%), lignin, cellulose (19,73%), hemicellulose (6.33%) and vitamins, also in the inorganic fraction has amounts of sulfates, oxalates, carbonates and silicates (CYPRIANO; LOPES DA SILVA; TASIC, 2018) and may be used in biotechnological processes as substrate to microbial growth because of its rich composition.

Currently, there is a tendency for the growth and development of agro-industrial processes, therefore, increase in production, in addition to residue generation growth, making the creation of suitable techniques to upgrading residues, mainly when these wastes are used to produce pharmaceutical products (ADETUNJI et al., 2022). The reuse of residues is centered on discard utilization and reduction trends, contributing to solving economic and environmental difficulties.

*B. atrophaeus* is a reclassification of B. subtilis gram-positive bacteria known as a producer of extracellular enzymes and spore-forming, which allows the utilization of *B. atrophaeus* as a biological indicator in thermal processes (SELLA et al., 2014a). This microorganism has important characteristics such as water-holding capacity, amorphous nature, and heat resistance (CHAMBI et al., 2022) and can be applied as more sustainable bioproducts in alternative culture medium (SANTO et al., 2022a).

This work aimed to study the orange juice bagasse as a substrate for developing *B. atrophaeus* spores to be applied in industrial and health practices.

## MATERIALS AND METHODS

### MICROORGANISM AND MEDIA

*B. atrophaeus* ATCC 9372 spores were kept in suspension and stored at 4°C until use. The media used were Plate Count Agar (PCA, Oxoid, Basingstoke, Hampshire, England) for spores' development as maintained media and Tryptic Soy Broth (TSB, Oxoid, Basingstoke, Hampshire, England) as purposed liquid media. A *B. atrophaeus* ATCC 9372 spore suspension was prepared using a previous culture maintained at the laboratory. An aliquot of 10.0 mL of homogenized suspension was placed in a test tube and heated to 80 °C for 10 min, followed by immersion in water with ice, being transferred to a Roux flask containing plate count agar surface (Oxoid, Basingstoke, Hampshire, England) at pH 7.2, and incubated into a B.O.D. at 37° C for 6 d (model 347, Fanem, São Paulo, SP, Brazil). After this period, the spore´s culture was harvested using glass beads in 0.02 mol.$L^{-1}$ calcium acetate solution, adjusted to a final pH of 9.7 with 0.14% (w/v) calcium hydroxide solution, and stored under refrigeration at 4°C (DAS NEVES et al., 2007).

## PREPARATION OF ORANGE SUBSTRATE

Fresh oranges (*Citrus sinensis L.*) were purchased from a local market in São Paulo, SP, Brazil. Fruits were rubbed under water, submerged into 0.1% (v/v) peracetic acid solution for 60 min, and rinsed. The bagasse obtained after juice extraction by fruit processor (BRCT, Britania, China), separating juice from bagasse (peel, seeds, and pulp), was frozen at -18° C until the moment of use.

To prepare the orange bagasse media, the amount of 1.0 g, 2.5 g, 5.0 g, 10.0 g, and 20.0 g was transferred to a 250 mL Erlenmeyer, containing 100 mL of deionized water and then sterilized at 121°C for 20 min. Each media from a different bagasse concentration was filtered through gauze-originating media for the cultivation of spores.

## *B. ATROPHAEUS* CULTIVATION INTO ORANGE BAGASSE MEDIA

An aliquot of 1 mL of *B. atrophaeus* suspension, previously homogenized, was inoculated in a 250 mL Erlenmeyer containing 100 mL of TSB and incubated at 37° C and 2g on an orbital shaker (TE-420, Tecnal, Piracicaba, SP, Brazil) for 24h. After this period, 10.0 mL of this culture was centrifuged at 5.000 rpm and 4°C for 20 min (5810 R, Eppendorf, Germany), separating the cell pellet from the supernatant. The pellet was resuspended with 10.0 mL of sterile water, and the inoculum biomass concentration was adjusted to 0.1 $g.L^{-1}$ by measuring the optical density (OD) at 600 nm (UV1650PC, Shimadzu, Kyoto, Japan) using calibration curve: Absorbance = 2.441*[*B. atrophaeus* ($g.L^{-1}$)]/ 0.2584 $R^2$= 0.98.

Each 100 mL of orange bagasse media was inoculated with 0.1 $g.L^{-1}$ of *B. atrophaeus* in a 250 mL Erlenmeyer and incubated on an orbital shaker at 37° C and 2g for 3 days and 6 days, using TSB as standard media. After cultivation, an aliquot of 10.0 mL was centrifuged at 4°C, 2,240 g for 20 min, separating cells from supernatant, for posterior analysis.

## pH DETERMINATION

The media pH was determined before and after the cultivation using a pH meter (pH 210, Coleman, Santo André, SP, Brazil) previously calibrated at pH 4.0 and 7.0 with standard solutions.

## CELL GROWTH DETERMINATION

Cell growth was determined by measuring the optical density (OD) at 600 nm, using a biomass of *B. atrophaeus* calibration curve: Absorbance = 2.441*[*biomass* ($g.L^{-1}$)]/ 0.2584, $R^2$= 0.98.

## DETERMINATION OF SPORE CONCENTRATION

An aliquot of 5 ml of each culture was treated at 80oC for 10 min and serially diluted in NaCl 0.9% (p/v). A sample of 1.0 mL of each dilution was transferred to Petri plate, and 20.0 mL of PCA were added. The plates were incubated at 37°C for 48 hours, and colonies were counted. The same method was used for total cell determination, proceeding without the heating step.

## GLUCOSE DETERMINATION

The glucose concentration of each media was determined by an enzymatic test (Wiener Lab, Rosario, Argentina). Aliquot of 10 µL was added in 1.0 mL of reagent solution containing glucose oxidase (GOD) and peroxidase (POD) enzymes, reacting at 37°C for 10 min (model 521, Nova Ética, Piracicaba, SP, Brazil) and sample coloring reading was performed at 505 nm. The glucose concentration was determined using the calibration curve: $Abs_{505nm}$ = 0.3698*[glucose ($g.L^{-1}$)] + 0.1139, $R^2$ = 0.995.

## REDUCING SUGARS DETERMINATION

The amount of sugars was determined by the colorimetric method of Somogyi-Nelson, with early hydrolyses of sucrose (MALDONADE *et al.*, 2013), using the calibration curve: Abs 540 nm = 1.3371*[glucose ($g.L^{-1}$)] + 0.0537, $R^2$ = 0.996.

## DECIMAL REDUCTION TIMES DETERMINATION

Decimal reduction times ($D_{Tr}$-value, min), the interval of time required to reduce one decimal logarithm of the initial spore population at the reference temperature, were determined from the negative reciprocal of the slopes of the regression lines using the linear portions of the survivor curves ($log_{10}$ population *vs* time of exposure at a constant temperature) a 102°C.

## CENTRAL COMPOSITE DESIGN

The central composite design (CCD), which uses the response surface methodology (RSM), was utilized to evaluate the relationship between the orange bagasse amount ($x_1$) and medium volume ($x_2$) in a $2^2$ factorial model using four assays in the classic level (±1.000), plus four assays with 45° rotational points (±1.414) and six replicates at the center point (n = 6), which led to 14 experiments, analyzed using Minitab statistical software®.

## RESULTS AND DISCUSSION

The highest biomass achieved was 1.700 g. $L^{-1}$ (± 0.014) in 10.0 g of orange bagasse after 6 days, 5.55 times higher than TSB 0.306 $g.L^{-1}$ (± 0.009). In 3 days of cultivation, the highest biomass was 0.973 (± 0.025) $g.L^{-1}$ in a bagasse concentration of 20.0 g, a value 20.8% higher than the standard medium 0.786 (± 0.009) $g.L^{-1}$ (Table 1).

After 3 days of cultivation, the highest biomass of 0.973 (± 0.025) $g.L^{-1}$ in 20.0 g of bagasse was 20% higher than in TSB (0.786 (± 0.009) $g.L^{-1}$. Using 10.0g and 20.0g of bagasse, $2.63 \times 10^7$ and $3.00 \times 10^6$ spores. $mL^{-1}$ were obtained, respectively (Table 1), reinforcing that nutrients are still disposable for cell maintenance, not for the development of spores (KHAN et al., 2009).

There was maximum sporulation using 5.0g of bagasse after 3d ($4.70 \times 10^8$ spores. $mL^{-1}$, pH 8.22 (± 0.13)), same sporulation condition after 6 days incubation. ($3.85 \times 10^8$ spores. $mL^{-1}$, pH 8.13 (± 0.02)) (Table 1). SELLA et al. (2014b) used sugarcane bagasse as a cultivation medium for *B. atrophaeus*, which provided good spore production between ($10^7$ and $10^9$) spores. The sporulation is favored by nutrient depletion in the medium with 6 days of incubation, where in all evaluated conditions, the concentration of $10^8$ spores. $mL^{-1}$ was reached. In contrast, in standard medium TSB, the spore concentration was $10^6$ spores. $mL^{-1}$, and $10^2$ cells remained in the vegetative state.

In standard medium, after 10h of cultivation, pH 6.86 (± 0.01), the difference between total cells and spores was also of three logarithmic cycles, with populations of $1.87 \times 10^8$ CFU.ml-1 and $1.90 \times 10^5$ spores. ml-1, respectively. At same time intervals of cultivation, the reduction in the number of total cells can be observed, which may be related to the population of *B. atrophaeus* in different stages of development, to the autolytic tendency towards the reduction of carbon source (MONTEIRO et al., 2014; SANTO et al., 2022a).

The initial amount of sugars was 2.100 (± 0.003) $g.L^{-1}$ in orange bagasse, considering total reducing sugars (ART) and 1.729 (± 0.012) $g.L^{-1}$ in TSB, considering only the glucose present in the medium. According to figures 1 and 2, it was observed that the sugar concentration after 4 hours of cultivation was twice higher in orange bagasse than in TSB, being 1.714

| bagasse (g) | pH medium | 3 days of cultivation | | | 6 days of cultivation | | |
|---|---|---|---|---|---|---|---|
| | | biomass ($g.L^{-1}$) | pH | spores. $mL^{-1}$ | biomass ($g.L^{-1}$) | pH | spores. $mL^{-1}$ |
| 1.0 | 4.9 | 0.487 (±0.015) | 8.19 (±0.31) | $1.30 \times 10^8$ | 0.291 (±0.007) | 8.81 (±0.03) | $1.48 \times 10^8$ |
| 2.5 | 4.7 | 0.293 (±0.017) | 8.35 (±0.03) | $2.13 \times 10^8$ | 0.252 (±0.008) | 8.85 (±0.01) | $2.85 \times 10^8$ |
| 5.0 | 4.6 | 0.710 (±0.007) | 8.22 (±0.13) | $4.70 \times 10^8$ | 0.925 (±0.017) | 8.88 (±0.02) | $3.85 \times 10^8$ |
| 10.0 | 4.5 | 0.507 (±0.005) | 7.46 (±0.04) | $2.63 \times 10^7$ | 1.700 (±0.014) | 8.13 (±0.02) | $2.12 \times 10^8$ |
| 20.0 | 4.5 | 0.973 (±0.025) | 5.91 (±0.11) | $3.00 \times 10^6$ | 0.985 (±0.061) | 5.48 (±0.07) | $3.75 \times 10^8$ |
| TSB | 7.1 | 0.786 (±0.009) | 8.61 (±0.01) | $6.95 \times 10^6$ | 0.306 (±0.009) | 9.04 (±0.02) | $3.05 \times 10^6$ |

**Table 1.** Values of pH, biomass (g/L) and spores of *B. atrophaeus* after 3 and 6 days of cultivation in orange bagasse media and TSB.

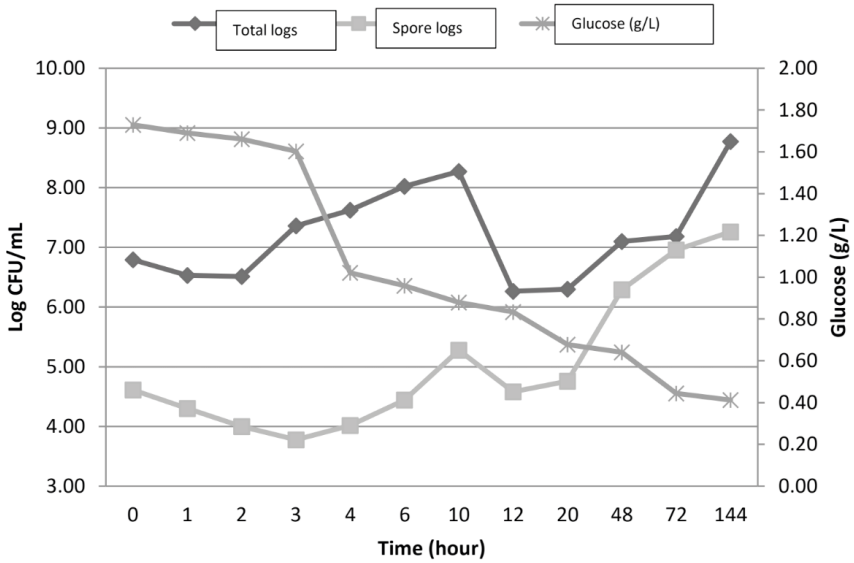


**Figure 1:** Growth curve of *B. atrophaeus* spores in TSB: (°) Log of vegetative cell concentration (CFU/mL); (°) Log of spore concentration (CFU/mL); (°) Glucose (g/L).

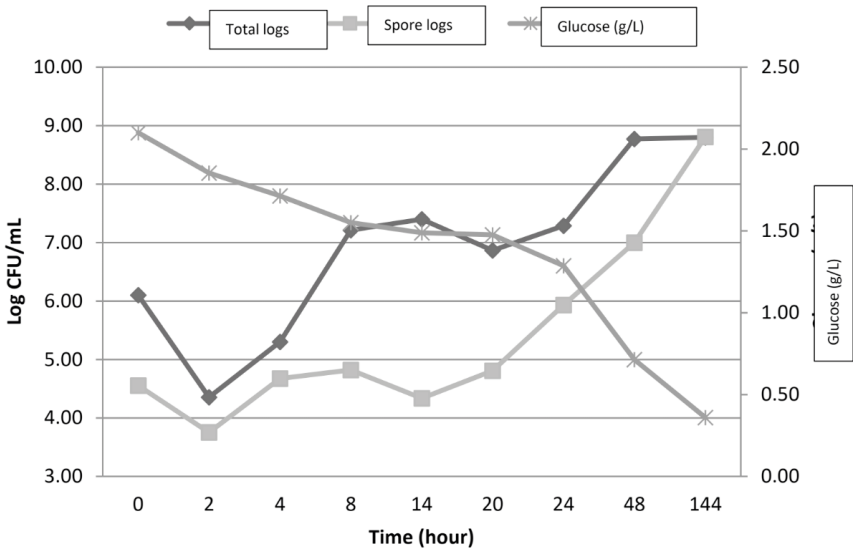


**Figure 2:** Growth curve of *B. atrophaeus* spores in 5.0 g of orange bagasse/100 mL of water: (°) Log of vegetative cell concentration (CFU/mL); (°) Log of spore concentration (CFU/mL); (°) Glucose (g/L).

(± 0.015) $g.L^{-1}$ and 0.880 (± 0.010) $g.L^{-1}$, respectively. This difference was maintained until approximately 20 hours of cultivation (1.476 (± 0.114) $g.L^{-1}$ and 0.663 (± 0.019) $g.L^{-1}$), approaching after 3d of cultivation, with 0.713 (± 0.009) $g.L^{-1}$ and 0.665 (± 0.389) $g.L^{-1}$. These profiles show that the consumption of glucose by the microorganism in bagasse is slower than in the standard medium since it must direct energy to cleave the total reducing sugars such as fructose and sucrose, adapting to the medium with the synthesis of enzymes needed to start the multiplication process (DAS NEVES *et al*., 2007). After 20h of cultivation, glucose consumption twice as much in TSB as in bagasse produced 60% more biomass after 4 hours, 30% more after 8 h, and reaching maximum values of 1.223 g. $L^{-1}$ in bagasse and 0.917 g. $L^{-1}$ in TSB (Figure 1 and 2).

The thermal resistance of spores was expressed as decimal reduction time (D value) determined at 102 °C using sodium chloride 0.9% (w/v) as vehicle (Table 2). Thermal, expressed in terms of decimal reduction time, is the main feature of the biological indicator and is defined by intrinsic and extrinsic factors such as cell growth, spore formation medium, temperature, cultivation time, the water content of spore, degree of mineralization, cortex, and spore cover structure (VESSONI PENNA *et al*., 2000).

| CCD | Decimal reduction (min) | |
|---|---|---|
| | 3 days | 6 days |
| DE1 | 2.07 | 2.03 |
| DE2 | 2.04 | 2.85 |
| DE3 | 2.57 | 1.64 |
| DE4 | 1.76 | 3.98 |
| DE5 | 1.78 | 1.99 |
| DE6 | 2.71 | 2.46 |
| DE7 | 1.59 | 1.43 |
| DE8 | 0.92 | 1.61 |
| DE9 | 1.46 | 1.37 |
| DE10 | 1.32 | 2.48 |
| DE11 | 1.54 | 1.58 |
| DE12 | 1.09 | 1.34 |
| DE13 | 1.08 | 2.08 |
| DE14 | 1.45 | 1.93 |
| TSB | 1.10 | 1.93 |

**Table 2.** Thermal resistance (decimal reduction – D), at 102ºC, of *B. atrophaeus* spores after 3 and 6 days of cultivation in orange bagasse media (5%), and standard media TSB, according to central composite design - CCD.

Decimal reduction times determined ranged from $D_{E8}$ = 0.92 min to $D_{E6}$ = 2.71 min for 3d of cultivation and from $D_{E12}$ = 1.34 min to $D_{E4}$ = 3.98 min for 6d of cultivation, similar values or higher than the D values determined for TSB spores of $D_{3d}$ = 1.10 min and $D_{6d}$ = 1.03 min (Table 2). The higher thermo-resistance presented by spores cultivated for 6 days corroborate with the previous studies (SELLA *et al*., 2014a), evaluated *B. atrophaeus* production by solid state fermentation using soybean residues and sugarcane bagasse for 9d, where they obtained a maximum yield of 1.90 x $10^{10}$ spores. $ml^{-1}$, with thermal resistance in dry heat of $D_{160C}$ = 5.2 min. By performing the regression analysis of the thermal resistance (TR) of spores of 3 days and 6 days, as a function of bagasse concentration, medium volume and incubation time, the following equation was obtained:

$$[TR] = \{0.904 + 0.0169 * [\text{bagasse (g)}] + 0.00212 * [\text{volume (ml)}] + 0.128 * [\text{time}$$

(d)]}, $R^2 = 0.111$, $p = 0.024$

By analyzing the behavior of spore thermal resistance only in the test group with fixed incubation time, the following equations were obtained by regression:

[RT 3d] = {1.59 - 0.0372 * [bagasse (g)] + 0.00456 * [volume (ml)]}, $R^2 = 0.149$, $p = 0.043$

[RT 6d] = {1.38 + 0.0710 * [bagasse (g)] - 0.00031 * [volume (ml)]}, $R^2 = 0.218$, $p = 0.008$

Although the thermal resistance of spores has a linear relationship between bagasse concentration, medium volume and incubation time, these models presented low coefficient of determination, which denotes a weak correlation model, being not ideal to correlate these studied parameters with thermal resistance. The D-values obtained at 102 °C under all conditions of the experimental design attest to the thermal resistance of *B. atrophaeus* ATCC 9372 spores cultivated in orange juice processing residues to be used as bioindicators of thermal processes.

The maximum spore production was $1.73 \times 10^9$ spores. $mL^{-1}$ and $5.75 \times 10^9$ spores $mL^{-1}$ after 3 days and 6 days of cultivation. The statistical study based on a $2^2$-point star-shaped factorial experimental design $2^2$ model presented the linear correlation: Spores = {-1.15 + 0.0303 * [bagasse (g)] - 0.00611 * [volume (ml)] + 0.611 * [time (d)]}, $p = 0.000$, $R^2 = 0.452$, with time ($p = 0.000$) being the most influential factor in spore growth and maturation. with the decimal reduction values ranging from: $D_{E8} = 0.92$ min to $D_{E6} = 2.71$ min and from $D_{E12} = 1.34$ min to $D_{E4} = 3.98$ min after 3d and 6d of cultivation at 102 °C, similar to data found in literature (SELLA *et al.,* 2014b; FLAX, B. et al., 2022).

## CONCLUSIONS

The culture media prepared from the orange juice processing residue proved viable for *B. atrophaeus* ATCC 9372 growth once biomass and spore's production were equal or greater than those obtained using the standard medium, adding value to this residue to obtain products of pharmaceutical and industrial interest. All spores obtained presented appropriate thermal resistance for bioindicators of thermal processes.

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