Beni-Suef University Journal of Basic and Applied Sciences

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Use of agro-industrial bio-waste for the growth and production of a previously isolated *Bacillus thuringiensis* strain

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Abstract

Background *Bacillus thuringiensis* (*Bt)* is a widely used biopesticide. The bioinsecticide based on *Bt* is obtained by fermentation, but the substrates currently used for its production constitute ingredients of high commercial value. In this context, the use of agro-industrial residues as substrates is an alternative to make the fermentation process viable on a large scale, in addition to minimizing environmental problems and contributing to the destination of these residues for biotechnological purposes.

Results In the first part of this study, a previously isolated spore forming soil bacteria (*Bv5*) harboring and expressing a novel *cry 8A* gene was confirmed as *B. thuringiensis* based on its morphological characteristics, Gram staining, scanning electron microscopy (SEM) and genome sequencing. *Bv5* was established as a Gram-positive spore forming bacteria with ellipsoidal spores and small round toxins. *Bv5* genome comprised of the 5.30 Mb chromosome and two megaplasmids of 450 kb and 261 kb, respectively, with *cry 8A* gene located on the smallest megaplasmid. In the second part of the study, the physiological profile of the *Bv5* strain during fermentation in different agro-industrial biowastes (cassava wastewater, orange pulp wash and whey) was analyzed. The fermentation experiment was divided into two stages. In the first stage, the agro-industrial waste with or without salts with the best results for biomass, spores and proteins production was selected. In the second stage, the effect of the selected medium in original and diluted form with the C:N balance was evaluated, in two different fermentation times (72 h and 96 h). Pulp wash enriched with salts was selected as the most suitable medium for the growth of *Bv5* strain in the first stage. In the second stage pulp wash (without dilution) with the addition of salts, and with nitrogen supplementation, was considered the best for cell growth, spore and toxin production by *Bv5*.

Conclusions To conclude, our study provide a new alternative for bio-waste from the orange juice industry, as well as potential culture medium for the *Bt* commercial scale production.

Keywords *Bacillus thuringiensis*, Biopesticides, Bio-waste, *Cry* gene, Fermentation

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1 Background

The ubiquitous *Bacillus thuringiensis* (*Bt*) is a Gram-positive rod-shaped bacterium, classified in the Bacillaceae family [33]. It is the most successful and widely studied biopesticide due its highly effective biological activity against insects' pests of agricultural and medical importance [2, 6, 30, 54] including the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, and many Acarina, protozoans, trematodes, and nematodes [38, 57]. In addition, *Bt* is also known for other important activities such as plant growth promotion, bioremediation, biosynthesis of metal nanoparticles, production of polyhydroxyalkanoate biopolymer, and anticancer activities [29, 44]. *Bt* genes have also been used to produce transgenic varieties of maize, cotton and soya bean, protecting these crops against approximately 30 major coleopteran and lepidopteran pests [22, 56].

The principal insecticidal activity of *Bt* is associated with the sporulation phase, forming crystalline delta-endotoxins, known as *cry* and *cyt* (insecticidal pore forming proteins). The parasporins, S-layer, *vip*, and *sip* are examples of other important proteins produced by *Bt* [20, 33].

Bt-based bioinsecticide are mainly produced by submerged fermentation using batch cultivation [32], however, fed-batch cultivation, solid-state and semi-solid-state fermentation are also utilized [32, 41, 59]. The raw material used in the fermentation process usually represents 35–59% of total cost. Hence, reducing the production costs is of great importance, both from an economic and practical point of view [39]. The use of industrial substrates used in its production generally increase the final price of *Bt*-based products. The possible replacement of synthetic fermentation media by equally effective natural raw materials, especially the use of agro-industrial waste as substrates is a promising as well as an environmentally sustainable option with positive economic impacts [15, 24].

Manipueira (a residual wastewater from cassava processing) is an agro-industrial by-product rich in carbohydrates and mineral salts that is generated in large quantities during the production of cassava flour, being considered a very attractive substrate for biotechnological processes [13]. Similarly, 85% approximately of the total volume of milk used in the manufacture of cheese is discarded as whey. Whey is rich in minerals, sugars and proteins and its inappropriate discard can result in serious environmental problems [27].

Brazil is one of the major producers of orange worldwide and the largest supplier of orange juice, followed by China, European Union, and United States [49]. On an average 34% of production is transformed into juice, but in large producing countries such as Brazil and the United States, this percentage reaches 90%. Pulp wash is a waste from the orange juice industry generated during the process of obtaining the orange juice.

Inadequate disposal of industrial waste, in addition to creating potential environmental problems, represents losses of raw materials and energy. Thus, adding value to these by-products (which are often region-specific), by using them as alternative low-cost substrates for obtaining products of high commercial value is of economic, social, scientific, and technological interest also resulting in a circular economy [23, 46]. Moreover agro-industrial wastes are often region-specific, and using them for *Bt* production allows for the utilization of local resources. The main objective of this work was to evaluate the use of these agro-industrial residues (Manipueira, Whey and Pulp wash) from cassava, dairy and fruit-based small industries as substrate for the growth and production of a new *Bacillus thuringiensis* (*Bv5*) isolate.

2 Methods

2.1 Organism, medium and culture conditions

The *Bacillus thuringiensis* (*Bt*) *Bv5* strain isolated in a previous work was used in this study [4]. After growth in standard medium at 30 °C for 72 h for sporulation, sterile filter discs were moistened with the cell-spore mixture, dried at room temperature, and stored at − 20 °C until further use. The bacterial cell-spore mixture was lyophilized after washing two times with cold distilled water and stored at − 20 °C until further use.

2.2 Optical and scanning electron microscopy (SEM)

Bv5 was submitted to the Gram and Coomassie brilliant blue (CBB) staining to confirm group classification and routine visualization of proteins, using light microscopy. For SEM, previously lyophilized sporulated mixture were re-suspended in 1 mL of ddH2O and an aliquot of 20 µL was used to prepare a bacterial smear on coverslip, which was dried in oven at 47 °C for 12 h, before gold sputtering (Bal-Tec, SCD 050) and observation of the ultrastructural features through SEM (JEOL, JSM-IT200LA).

2.3 Genome sequencing

Genomic DNA was isolated from the *Bv5* strain for Nanopore long-read and Illumina short-read sequencing. A 5 mL overnight culture of *Bv5* was spun down at 5000 g. The pelleted cells were re-suspended in 160 µL of QIAGEN Buffer P1 with 2 mg of lysozyme and incubated at 37 °C for one hour, then DNA was isolated from the sample using the QIAGEN MagAttract HMW DNA kit, according to the manufacturer's instructions, with minor modifications. During the final elution of DNA from the magnetic beads, beads were incubated in 50 µL of TE buffer for 10 min, and elution was

performed twice, with the eluate collected together in one tube. Output DNA concentration was measured using a Qubit high-sensitivity DNA assay. DNA was sent to the McMaster Genomics Facility for sequencing on Nanopore long-read and Illumina HiSeq 2500 with 2×250 bp reads. 2 µg of DNA was re-suspended in 50 µL of TE buffer, and size selection was performed according the ONT SPRI Size Selection protocol. Size selected DNA was then barcoded and sequenced on a ONT MinION R9.4 flowcell using the LSK-109 Ligation sequencing kit, along with 3 other samples. Long-read sequencing was performed for 36 h with MinKNOW version (20.10), without live basecalling. Sequenced reads were uploaded to the Compute Canada Graham cluster and basecalled with Guppy version (5.0.11) using the R9.4 high-accuracy flip-flop model.

Illumina sequencing reads were trimmed and filtered using fastp [11], and error-corrected using Pollux [31]. Nanopore long-reads were demultiplexed and adapter trimmed with porechop, with matching barcodes required at both ends of the read. Reads shorter than 5 kb or with a mean quality score below 12 were filtered out with Nanofilt [12]. Genome assembly was performed using error-corrected and filtered reads using Unicycler version (0.5.0) in hybrid assembly mode. Settings were left as default, except the error-correction was disabled, and a custom-compiled version of spades with support for K-mers up to 251 bp was used for the Illumina portion of the assembly. Genome annotation was performed using DFAST version 1.6.0 [51] with a custom-compiled database of proteins from 48 *B. thuringiensis* genomes found on NCBI. To improve the number of useful annotations of the putative proteins, all entries labeled "hypothetical protein" or "putative protein" were removed from the database before annotation of the *Bv5* genome.

2.4 Agro-industrial wastes

The manipueira (M) and whey (W) were obtained from small agro-industries of cassava and cheese production, respectively, located in the Sergipe state, Brazil. The pulp wash (Pw) was obtained from the TropFruit Nordeste SA (Estância, Sergipe, Brazil). Pulp Wash is a product obtained from the residues of orange juice extraction. After the pulp has been separated, it is thoroughly washed with water to extract the remaining juice. The concentrated and frozen pulp undergoes pasteurization, evaporation and filtration to achieve a concentration level of 65°BRIX. All the residues used in this study were preserved at − 20 °C. Before use they were filtered to remove the coarse particles, pH was adjusted to 7, and sterilized.

2.5 Fermentation assays

The fermentation experiments were performed in two stages. In the first stage, the agro-industrial wastes were used in their original (undiluted form), with or without addition of mineral salts: KH_2PO_4 0.3%, K_2HPO_4 0.1%, and $MgSO_4$ 0.4% [21] and compared to the standard medium. In the second stage, the substrate selected in first fermentation stage was evaluated in original (PwS + N) and diluted form in 1:1 and 3:1 dilutions (PwS + N 1:1 and PwS + N 3:1) with distilled water and supplemented with 11.52 g L^{-1} of ammonium nitrate (NH_4NO_3) to obtain a C:N ratio of 7:1 [60]. The selected substrate in the original form without nitrogen supplementation (PwS) was used as a control. The seed culture was prepared by first inoculating a filter paper disk in 5 ml standard medium (28 °C, 24 h, 200 rpm), which was then added to 100 ml of standard medium and cultivated for 24 h at 28 °C and 200 rpm. 4 mL of this seed culture was used as inoculum for starting the fermentations in flasks containing 100 mL of the sterilized substrates at pH 7. The fermentations were carried out in triplicates at 28 °C, 200 rpm for 72 h in the first step and at 28 °C, 200 rpm for 72 or 96 h in the second step.

2.6 Fermentation parameters

2.6.1 Biomass

At the end of each fermentation, the cultivation media were centrifuged in Falcon tubes (50 mL) at 4 °C, 10,000 rpm for 20 min. The pellet was washed twice with cold distilled water (4 °C, 10,000 rpm for 20 min) and stored in the freezer at − 20 °C until lyophilization process (72 h) for biomass determination by using the following equation:

$$\begin{aligned}&\text{Dry Biomass Production}\,(\text{g})\\&=\text{Lyophilized Pellet Weight}\,(\text{g})\\&\quad-\text{Empty Eppendorf Weight}\,(\text{g})\end{aligned}$$

2.6.2 Spores and viability

Serial dilution was performed using 0.02 g of lyophilized pellet re-suspended in 10 ml of distilled water. The diluted material was used for determination of spores ml^{-1} using Neubauer chamber [3]. The sample obtained by serial dilution was also submitted to a thermal shock (80 °C for 12 min and 0 °C for 5 min), for eliminating the vegetative forms. An aliquot of 100 µL was spread with a Drigalski spatula over the entire surface of nutrient agar medium and incubated at 28 °C for 16 h. The colonies were counted and expressed in colony forming unit per mL (CFU ml^{-1}).

2.7 Total protein

Total protein was quantified by the Bradford method [8]. 0.02 g of the lyophilized pellet from each treatment was weighed and diluted in 500 µL of 50 mM NaOH and placed in a water bath for 15 min at 37 °C. Then, the samples were centrifuged at 13,000 rpm at 4 °C for 5 min. The pellet was discarded, and the supernatant was used for protein quantification, where 200 µL of the sample was mixed in 3 mL of Bradford's reagent and allowed to react for 10 min. After this period, 200 µL of each sample was placed in 96-well microplates for reading in a UV–vis spectrophotometer (Epoch 2 microplate reader, Bio Teck) at 595 nm absorbance.

2.8 Statistical analysis

The experimental design used for the fermentation assays was completely randomized (DIC), with three replications. The results were submitted to two-way analysis of variance (Two-way ANOVA) and the treatment means were compared by Tukey's test at the 5% probability level. For the analysis of the results, the software IBM SPSS Statistics v. 23 was used.

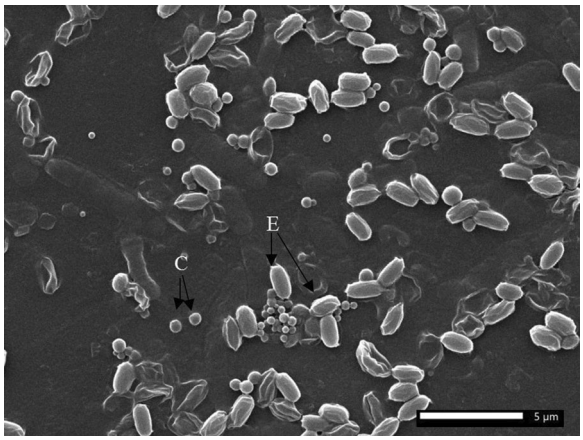

Fig. 1 Scanning electron microscopy images of the *Bv5* strain, showing ellipsoidal spores and round proteins not attached to the spores. (Captions: C, Crystals; E, spores)

3 Results

3.1 *Bv5* isolate

On nutrient agar *Bv5* grew as circular, white, opaque colonies with irregular margins which is like *Bt* colonies. Observations under light microscope (Gram staining followed by Coomassie blue staining) confirmed that the isolate *Bv5* was rod-shaped, Gram-positive spore forming bacteria, producing ellipsoidal spores with dense edges and small round rod-shaped spores. SEM was carried out with a sporulated culture to better visualize the spore and toxin morphology, showing *Bv5* ellipsoidal spores and small round toxins (Fig. 1).

3.2 Genome sequencing

After error-correction and filtering, a total of 1.88 Gb of Illumina short reads and 551 Mb of Nanopore long-read data were produced for the *Bv5* isolate. Assembly with Unicycler produced 3 circular contigs, comprised of the 5.30 Mb chromosome and two megaplasmids of 450 kb and 261 kb, respectively, with an overall GC content of 35%. The chromosomal and plasmid sequences were deposited in the GenBank with the accession numbers CP125661, CP125662 and CP125663. Gene annotation with DFAST identified 5886 open reading frames, as well 43 rRNAs, 107 tRNAs, and 3 CRISPRs. BLAST search identified the genome as *Bacillus thuringiensis*, with 99.95% identity to *Bacillus thuringiensis* strain *c25* complete genome (Accession Number: CP022345). The novel *cry* gene was found in a genomic neighborhood flanked by an IS150 gene cluster upstream and an IS605 family gene cluster downstream (Fig. 2). Genome characteristics of the *Bv5* strain are shown in Table 1.

3.3 Fermentation assays

The agro-industrial residues (manipueira, whey, and pulp wash) showed physiological differences in the behavior of *Bv5* strain. The fermentation assays as stated before were carried out in two stages. During the first phase, biomass (g), spores (spores ml^{-1} and CFU ml^{-1}) and total proteins (µg ml^{-1}) were determined in order to select the most suitable substrate (whey, manipueira or pulp wash), with or without addition of salts (Fig. 3). During the second phase, culture media selected in the first phase were

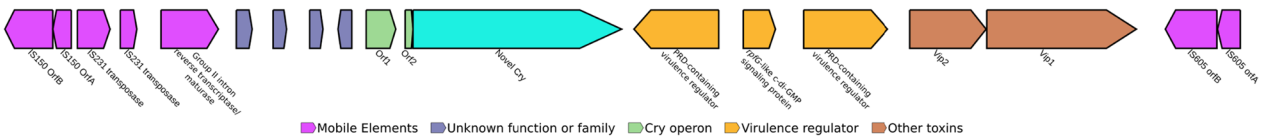

Fig. 2 Genomic context of the novel *cry* 8 gene. The region is flanked on either side by various mobile genetic elements (indicated in purple). Predicted genes without identified function or family are shown in grey, putative members of the *cry* operon in green, predicted virulence regulators in yellow, and the *vip1*/2 insecticidal toxins in brown. The arrow size is proportional to gene length, but gene spacing is not proportional to intergenic distance

Table 1 Genome characteristics of the BV5 strain

Features	Value
Genome size (bp)	6,055,894
Number of contigs	3
G + C content (%)	35.0
Shortest contig size	260,890
Median sequence size	499,711
Mean sequence size	2,018,630
Longest contig size	5,295,289
Total genes	6036
Protein-coding genes	5886
RNA genes	150
rRNAs	43
tRNAs	107
tmRNAs	1
CRISPR repeats	3
Genes with no function prediction	958
Genes with function prediction	4928

evaluated for the same parameters, but at different dilutions of the selected substrate and after adjusting the C:N ratio (Fig. 4). These growth and other evaluations were carried out after 72 and 96 h growth periods.

4 Discussion

In this work, *Bv5* ellipsoidal spores and small round toxins were recorded by SEM (Fig. 1). The investigation of the protein predictions identified one gene with an identical sequence to the previously identified novel *cry* gene [4], located on the smallest megaplasmid. The novel *cry* gene is located in a genomic neighborhood flanked by an *is150* gene cluster upstream and an *is605* family gene cluster downstream (Fig. 2).

Four gene predictions (light grey in Fig. 2) code proteins of unknown function, with no identified conserved domains; BLAST search identified only other *B. cereus* sensu lato predicted proteins with significant sequence similarity (data not shown). Two proteins are immediately upstream, and in the same orientation as the novel *cry* gene. The first, indicated as Orf1 in Fig. 2, is highly similar to the Orf1 found in the operon of *cry*8 proteins. Orf2 frame with the novel *cry* gene and separated by a single stop codon. Since it has no significant BLAST hits in the NCBI nr protein database, Orf2 may be the result of a nonsense mutation truncating the 5' end of the novel *cry* protein. Three genes with putative regulatory function in virulence are located downstream of the novel *cry*. Two genes encode proteins containing sugar phosphotransferase system (PTS) regulation domains (PRDs) and other domains similar to the PTS system transcriptional regulators [47], which have been found in regulating virulence factors in *B. cereus* sensu lato members [17]. The remaining gene codes for a protein containing the HD-GYP domain, which degrades c-di-GMP, an important signaling molecule for a variety of pathogenic bacteria [48]. Finally, two insecticidal toxicity related genes, *vip1* and *vip2* [26], are downstream of the regulatory proteins, followed by the two-member IS605 gene cluster.

In the first stage, whey with salts (SS) and manipueira with salts (MS) showed the best results with respect to biomass production with an average of 0.548 g and 0.509 g, respectively, with no significant difference between them (Fig. 3). The pulp wash with salts (PwS) (0.362 g) and whey without salts (S) (0.308 g) did not show significant differences between them, but showed differences when compared to the standard medium (0.114 g), indicating that they also favored the growth of the *Bv5* strain after 72 h of fermentation. The standard medium (0.114 g) and manipueira without salts (M) (0.288 g) showed the lowest biomass production.

The substrate pulp wash with salts (PwS) showed the highest value of spore formation (5.30×10^{11} spores ml^{-1}) at 72 h fermentation. On the other hand, in this period the standard medium produced 1.40×10^{8} spores ml^{-1}, presenting the lowest value of spore formation, not differing from the other substrates tested (MS, S, M and Pw).

With respect to the spore viability, measured by colony forming unit counting (CFU), the pulp wash with salts (PwS) showed the highest value of 1.81×10^{9} CFU ml^{-1}, followed by the whey without salts (S) (1.28×10^{9} CFU ml^{-1}). The PwS and S culture mediums presented differences between them and higher CFU values compared to the standard (3.5×10^{5} CFU ml^{-1}) and the other agro-industrial residues evaluated. A recent survey used *Bt* var. *tenebrionis* in an experimental design for culture media optimization in an effort to achieve high levels of CFU. The results showed maximum CFU of 4.85×10^{7} ml^{-1} in sugar cane molasses fermentation after 72 h. This result was achieved at the maximum carbon and minimum nitrogen sources, above 11%v and less than 2%v, respectively [42].

The manipueira without salts (M) and pulp wash with salts (PwS) produced highest concentrations of total protein, 0.955 µg ml^{-1} and 0.925 µg ml^{-1}, respectively. In contrast, the standard medium showed the lowest values of total protein (0.289 µg ml^{-1}). It is well established that the nutritional needs of *Bt* are variable and dependent on the strain type. This means that a specific substrate may have inhibitory action for other *Bt* strains, highlighting the need of defining the specific nutritional requirements for each strain studied. Besides that, *Bt* strains presents high genetic variability and different toxicity profiles [10]. The differential regulation of toxin proteins was earlier revealed in the same strain, showing the possibility of

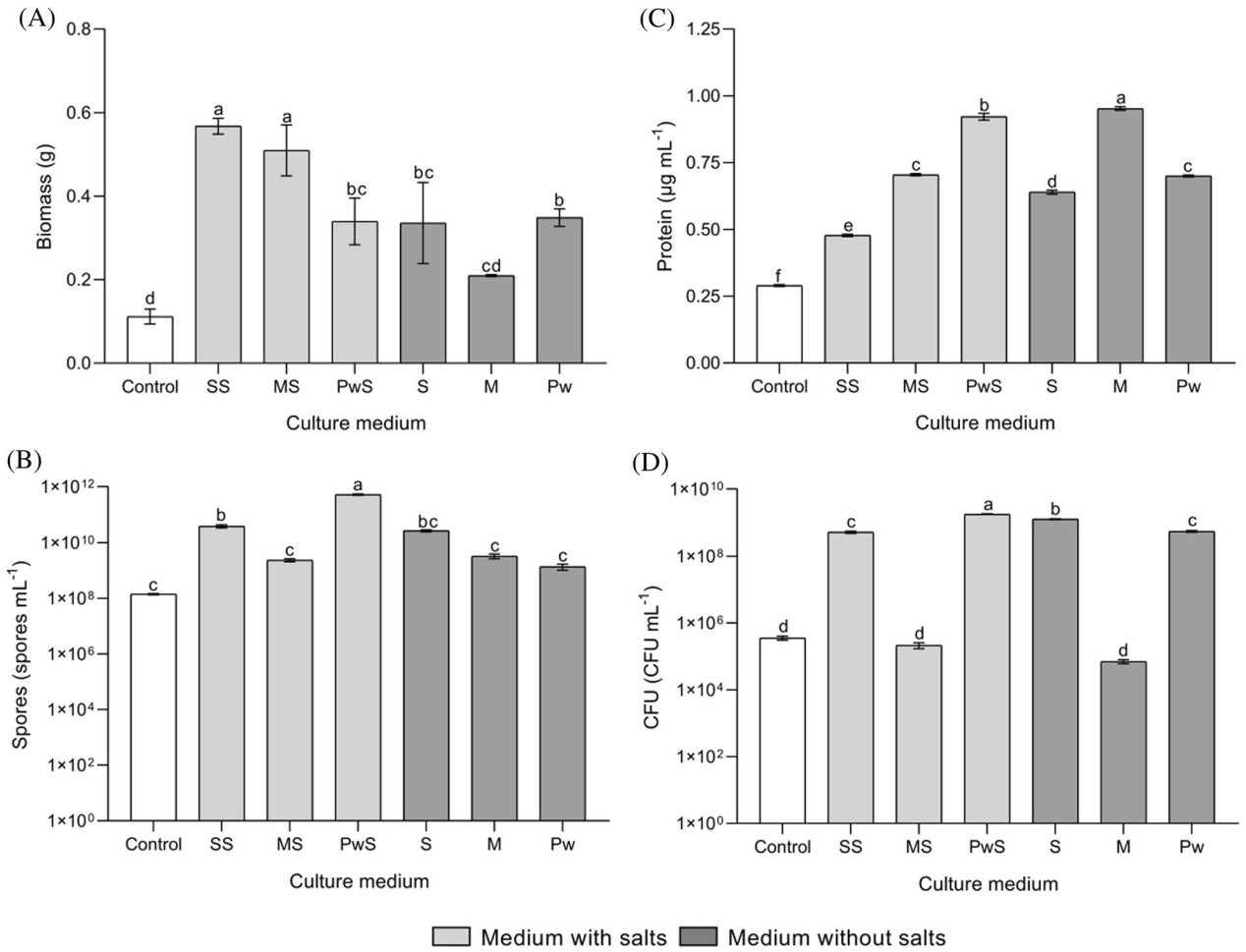

Fig. 3 Biomass production (**A**), spore count (**B**), quantification of crystal protein (**C**), and determination of CFU.mL^{-1} of *Bv5* strain (**D**), in different culture media, after 72 h of fermentation. Means followed by the same letter did not differ from each other by the Tukey test ($p < 0.05$). The error bar corresponds to the standard error. (Control) Standard medium; (SS) Whey with salts; (MS) Manipueira with salts; (PwS) Pulp wash with salts; (S) whey without salts; (M) manipueira without salts; (Pw) Pulp wash without salts

selection of medium ingredients for directed synthesis of toxin components [28]. Different types of proteases (metalloproteases, alkaline serine and cysteine proteases) encoded by genes such as nprA, epr, aprA are produced by *Bt*, which are crucial for its insecticidal activity against different insect orders [9, 14, 16, 37, 50]. These proteases are involved in *Bt* growth, sporulation and gene expression for the production of the insecticidal crystal protein. They influence the preparatory hydrolysis of the crystal proteins and contribute to the destruction of the insect defense mechanism [9]. In this respect, proteases are indicators of entomotoxicity and have been shown to be direct determinants of *Bt* growth [9]. These enzymes are regulated by specific genes that are activated in response to the presence of organic compounds in bio-waste [58].

Other genes associated with *Bt* growth and sporulation such as spo0A, spo0B, spo0F, sigE and sigK, are known to be induced by environmental factors such as temperature and nutrient availability, and play a crucial role in regulating and controlling the processes of growth and sporulation [25, 43, 55]. The alternative production of *Bt* could be enhanced by the use of agro-industrial residues as a nutrient source. These residues, which are rich in carbohydrates, amino acids and other organic compounds, can be used to promote bacterial growth and stimulate the expression of genes involved in toxin production [5, 34]. The use of agro-industrial residues as substrates for microbial growth is a sustainable and cost-effective strategy to maximize biomass, spore and toxin production. The presence of these organic compounds promotes bacterial growth and activates genes related to toxin production, leading to a higher production efficiency of these compounds, using materials that would be discarded [7, 35].

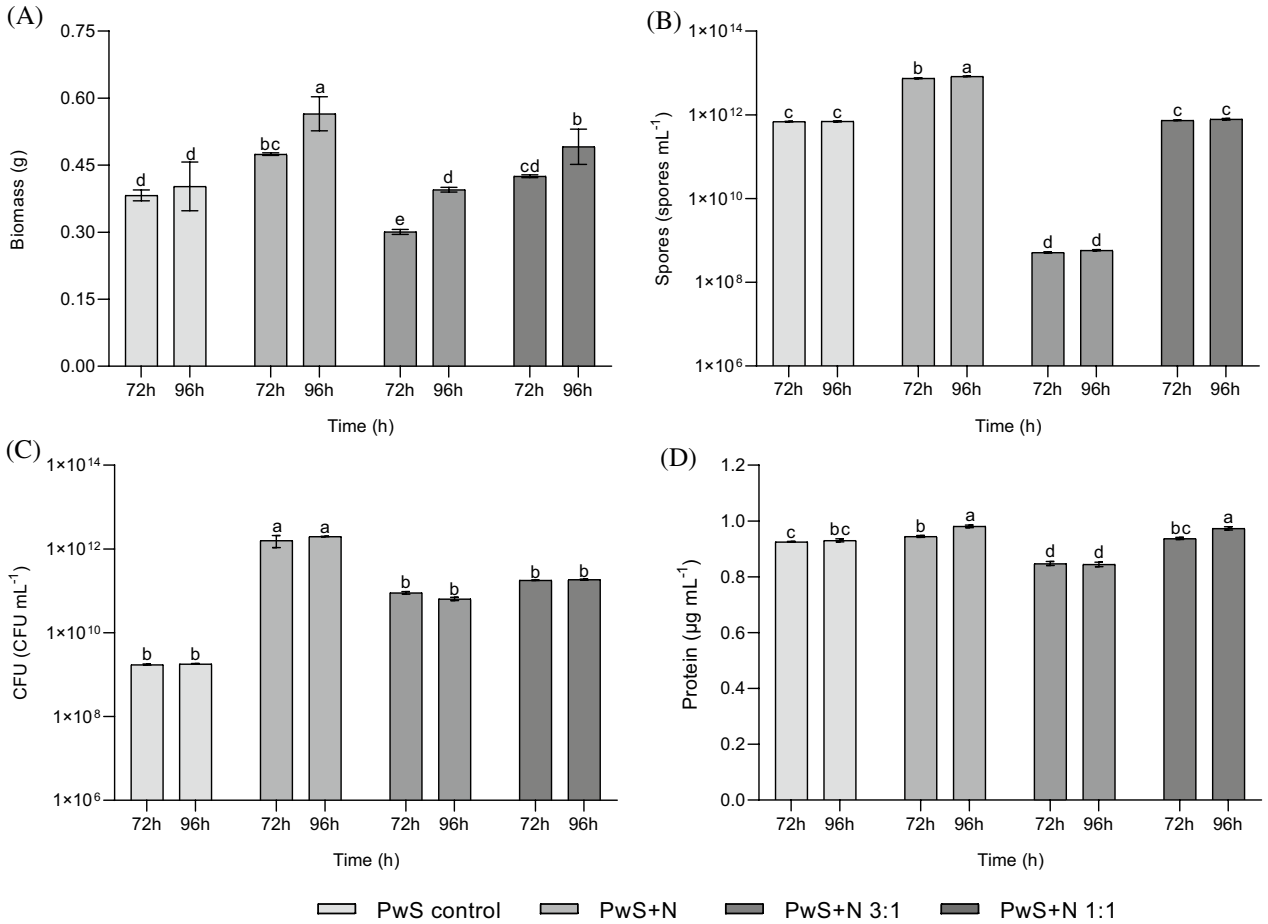

Fig. 4 Biomass production (**A**), spore count (**B**), determination of CFU.mL−1 of Bv5 strain (**C**), and quantification of crystal proteins production (**D**) by *Bv5* strain in different dilutions of pulp wash with salts with and without addition of nitrogen (ammonium nitrate), after 72 and 96 h and fermentation. Means followed by the same letter did not differ from each other by the Tukey test ($p < 0.05$). The error bar corresponds to the standard error. (PwS) Pulp wash with salts without nitrogen balance; (PwS + N) Undiluted pulp wash with salts and nitrogen balance; (PWS + N 1:1) 50% pulp wash + 50% water with salts and nitrogen balance; (PWS + N 3:1) 75% pulp wash + 25% water with salts and nitrogen balance

A wide variety of commercial culture media and industrial substrates have been tested for optimizing *Bt* production [15, 18, 24, 61] using wastes from starch industry, sewage plants, the poultry industry, slaughterhouses, agro-business and restaurants [15]. A recent study used a cost-effective liquid culture media based on agricultural raw materials (edible leguminous seeds, corn, wheat bran, dry palm, date pulps, and corn forages) and agro-industrial wastes (dry dates palm pits and olive mill solid wastes) for spores and delta-endotoxins production by a *B. thuringiensis* subsp. *Aizawai* strain [24]. The dry date palm extract was the most efficient medium for spore production and presented 1.43×10^{10} spores ml^{-1} after 72 h of incubation. The chickpea medium showed a production of 581 µg ml^{-1} delta-endotoxins, displaying the most efficient results [24]. Starch industry wastewater was also investigated for *Bt* kurstaki fermentation, presenting a high total cell count of 2.90×10^8 CFU ml^{-1}, viable spores of 2.4×10^8 CFU ml^{-1} and a maximum endotoxin concentration of 435 µg ml^{-1} after 48 h fermentation [36]. In another study, whey was used as a substrate for the production of protease by a *B. thuringiensis* strain. The minimum medium supplemented with whey and skim milk presented 6000 mg L^{-1} dry weight, in which 2600 mg L^{-1} total proteins were recorded within 72 h of fermentation [19].

Valicente et al. [53] analyzed tree different media to improve *Bt* growth. Medium 1 containing: LB, $FeSO_4$, $ZnSO_4$, $MnSO_4$, $MgSO_4$ (salts) and 0.2% glucose, medium 2 containing: 1.5% glucose, 0.5% soybean flour and salts, and medium 3 containing: 4% liquid swine manure and 0.2% glucose. Medium 1 enriched with salts showed the highest spore concentration, 1.4×10^9 spores ml^{-1} after 96 h of fermentation.

Pulp wash is an agro-industrial waste obtained via washing the machines used for orange juice extraction.

This substrate (Pw) contains large amounts of polysaccharides, such as cellulose, hemicellulose and pectin [40, 45], contributing as a carbon source, a fundamental requirement for the *Bt* fermentation process. The growth, sporulation and production of delta endotoxin by *Bt* besides carbon, also require nitrogen, potassium, and metal ions sources [28, 42, 52]. The presence of mineral elements and polysaccharides in the PwS may have contributed to obtaining the highest production of viable spores, both in evaluation by Neubauer chamber (5.30×10^{11} spores ml^{-1}) and by CFU counting (1.81×10^{9} CFU ml^{-1}). In addition, PwS also produced higher amounts of total proteins (0.925 µg ml^{-1}) and biomass (0.3620 g) compared to the standard medium.

In view of the results obtained in the first fermentation step, the salt-enriched pulp wash (PwS) was selected as the most suitable medium for the growth of the *Bv5* strain for the second fermentation experiment. The highest biomass yields were obtained by PwS + N, at both fermentation (72 and 96 h) times evaluated. At 96 h fermentation the PwS + N showed the highest biomass of 0.555 g, compared to all other treatments evaluated (Fig. 4). The fermentation time was important for the *Bt* growth, since PwS substrates reached highest biomass values at 96 h for all treatments.

The PwS + N showed the highest values of spores ml^{-1} of 7.48×10^{12} and 8.26×10^{12} at 72 and 96 h, respectively. The PwS control produced 6.97×10^{11} spores ml^{-1} at 96 h and did not show difference compared to the PwS + N 1:1 and PwS + N 3:1. When PwS + N was 1:1 or 3:1 diluted, lower sporulation was obtained even with NH_4NO_3 supplementation. The substrate PwS + N 1:1 showed the lowest values of spores ml^{-1} of 5.14×10^{8} and 5.92×10^{8} at 72 and 96 h fermentation, respectively.

The PwS + N also showed the highest values of CFU ml^{-1} (1.59 and 1.99×10^{12}) at 72 and 96 h. The CFU values from other diluted treatments and PwS control did not show difference between them at 72 and 96 h. A recent study used a *B. thuringiensis* strain for the growth under shaker conditions to produce insecticidal vegetative protein (*Vip3*). The combination of 20 g L^{-1} of glucose as carbon source and 15 g L^{-1} of soybean as nitrogen source (C:N ratio of 3.88) promoted the highest vegetative cell count of 9.5×10^{10} CFU at 24 h [2].

The PwS + N produced 0.981 µg ml^{-1} of total protein and did not show difference when compared to PwS + N 3:1 (0.974 µg ml^{-1}) at 96 h fermentation. The PwS + N 1:1 presented the lowest production of 0.844 µg ml^{-1} of total protein at 96 h.

Many different types of supplementations have been reported to optimize the medium including the use of soluble starch as carbon source from vegetable wastes, soya bean meal as nitrogen source and use of metal ions. Pan et al. [39] used a combination of these additives and showed the optimal composition of 6 g L^{-1} soluble starch, 6 g L^{-1} soya bean meal, 0.4 g L^{-1} Fe^{2+} and 0.4 g L^{-1} Al^{3+}, for the growth of a *Bt* strain. By-products rich in carbon and nitrogen as nutrient sources have been reported to improve *Bt* fermentation. The medium formulated with 1.0% of maize glucose and 3.0% of soy flour promoted the production of 4.39×10^{9} spores ml^{-1} and 39.3 g L^{-1} of dry biomass after 96 h fermentation [52]. The sugar cane molasses enriched with 200 mg L^{-1} of yeast extract also was earlier used to produce biopesticide from *Bt* var. kurstaki HD-1, showing results of 7.1×10^{11} CFU ml^{-1} of cell biomass at 24 h and 1×10^{12} CFU ml^{-1} of spore concentration at 48 h fermentation [1]. These studies reinforce that agro-industrial residues can be an excellent source to improve *Bt* fermentation which can be further improved with supplementation of nitrogen and carbohydrate and mineral elements, contributing to sustainable development. Nitrogen source regulation has been shown to have a significant control on the biosynthesis of toxin proteins [28].

In the present study, C:N balance in the proportion of 7:1 and the addition of KH_2PO_4, K_2HPO_4 and $MgSO_4$ improved the growth (0.555 g), sporulation (8.26×10^{12} spores ml^{-1}), spore viability (1.99×10^{12} CFU ml^{-1}) and production of crystal proteins (0.981 µg ml^{-1}) when pulp wash was used as a culture medium (PwS) (96 h). Pulp wash is a low-cost agro-industrial residue easily acquired from citrus agroindustry. Supplementation of the agro-industrial waste with the salts and nitrogen resulted in a suitable medium to produce *Bt*-based bioinsecticides.

5 Conclusions

Bt var *Bv5* vegetative and reproductive morphological features, e.g., ellipsoidal spore and small round toxins, were not affected in the conditions tested. *Bv5* genome matched with the other *Bt* genomes present in GenBank database and confirmed the presence of novel *cry* 8 gene cloned in an earlier study. Among the alternative substrates studied, pulp wash (without dilution) with the addition of salts, and with nitrogen supplementation, was the most efficient culture medium for cell growth, spore and toxin production by *Bv5*, thus providing a new alternative for the waste from the orange juice industry, as well as potential culture medium for the *Bt* commercial scale production. The development of a new *Bt* bioproduct based on agro-industrial waste is crucial to promote sustainability in agriculture and offers a more efficient alternative to meet the needs of the agricultural sector. Bioproducts from

agro-industrial waste reduce environmental impact, waste of resources and dependence on conventional raw materials.

Abbreviations

ANOVA	Analysis of variance
Bt	*Bacillus thuringiensis*
CBB	Coomassie brilliant blue
DNA	Deoxyribonucleic acid
M	Manipueira
Pw	Pulp wash
SEM	Scanning electron microscopy
W	Whey

Acknowledgements
Authors would like to thank Professor Brendan McConkey and Mark Lubberts, University of Waterloo, Canada, for their help in genome sequencing and genome annotations.

Author contributions
HMMS, CSV, CASV, FCAD, MVAB, RPMF and SJ contributed for the conceptualization, methodology, validation, formal analysis, investigation, and writing—original draft preparation. PS, EBS, SSD, MCM and SJ contributed for the methodology, supervision, writing—review and editing, project administration, resources, and funding acquisition. All authors have contributed substantially to the work. All authors read and approved the final manuscript.

Funding
This research was supported by CAPES and FAPITEC/SE through the scholarships and funding of the first and second authors. FCT—Fundação para a Ciência e a Tecnologia, I.P. supported EBS within the scope of the projects UIDP/04378/2020 and UIDB/04378/2020 of the Research Unit on Applied Molecular Biosciences—UCIBIO, and the project LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy—i4HB.

Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Authors confirm that no ethics approval and consent to participate is applicable for this work. No ethics issues are raised in this manuscript.

Consent for publication
All authors agreed with the final version of this manuscript and with the current submission.

Competing interests
The authors have no relevant financial or non-financial interests to disclose.

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Received: 7 August 2023 Accepted: 27 December 2023
Published online: 04 January 2024

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