Finanziato dall'Unione europea
NextGenerationEU

Ministero dell'Università e della Ricerca

Italiadomani
PIANO NAZIONALE DI RIPRESA E RESILIENZA

agritech
National Research Center for Technology in Agriculture

Evaluation of microbial populations encoding the endo-1,4-ß-glucanase – *bcsZ* gene for the isolation of new microbial strains from *Hermetia illucens* larvae.

I. Romano[1], M. Amato[1], M. Casartelli[2], L. Amoroso[1], G. Tettamanti[3], D. Bruno[3], G. Magaraci[1], M. Bonelli [2], V. Ventorino[1-4], O. Pepe[1-4]

[1]Department *of Agricultural Sciences, University* of Naples Federico II,

[2]Department of Biosciences University of Milan,

[3]Department of Biotechnology and Life Sciences University of Insubria,

[4]Task Force on Microbiome Studies, University of Naples Federico II, Naples, Italy

E-mail: Ida.romano@unina.it; olipepe@unina.it

SPOKE: 8.1.2: *Biosystems for obtaining bioproducts from lignocellulosic biomass and agro-industrial by-products.*

Abstract

As global demand for sustainable resources rises, research into biotechnological solutions for agricultural waste valorization intensified. Lignocellulosic biomass is an important resource that is not extensively utilized, which can be converted into useful bioproducts Traditional methods of biomass conversion, such as chemical and thermal processes, are often energy-intensive and environmentally damaging. In contrast, biological methods offer an eco-friendlier and more sustainable alternative.

Hermetia (H.) illucens, known as black soldier fly, emerged as a key player in this field due to its larvae's ability to process organic waste. The larvae's gut microbiome can break down lignocellulosic materials, making it a rich source of cellulolytic bacteria. These bacteria produce enzymes that degrade cellulose into simpler sugars, which can then be converted into biofuels, chemicals, and other valuable products. The aim of this study is to obtain new cellulolytic microorganisms from *H. illucens* larvae grown on alternative cellulose substrates such as hemp. To identify and confirm bacteria that produce cellulase, techniques including the use of enrichment media and semi-quantitative assays on carboxymethyl cellulose (CMC) agar, were employed. Additionally, molecular methods like quantitative PCR (qPCR) were used to further investigate the expression of *bcsZ* genes involved in cellulose degradation.

Integrating these biological processes into waste management systems can foster a circular economy by converting agro-industrial waste into renewable resources. This approach not only addresses waste disposal challenges but also supports the production of biofuels and other bioproducts, benefiting various industries and promoting environmental sustainability. Exploring novel microorganisms and their enzymatic capabilities holds promise for advancing sustainable industrial practices and enhancing the global bioeconomy.

Materials and methods

Hermetia illucens larval guts and larvae grown on hemp substrate were inoculated in two enrichment substrates (A1: CMC, soil extract, hemp powder, and larval extract, A2: *A. (A.) donax*, soil extract, hemp powder, and larval extract). Serial dilutions of these samples, taken at different time points (T0: 2 hours, T1: 7 days, and T2: 15 days), were plated on four media containing different sources of cellulose [1] (CMC, *Arundo (A.) donax*, hemp, Avicel) to select cellulolytic microorganisms (Figure 1). Cellulolytic activity of the new isolates was confirmed by semi-quantitative spot method on CMC agar stained with 1% Congo red [2-3] (Figure 2). Cellulase activity was calculated as the diameter of the cleaning halo zone subtracted by the colony diameter (Indices of Relative Enzyme Activity-ICMC). The purified isolates underwent to phenotype characterization based on colony morphology, microscopy shape, Gram staining, and catalase assays, and stored in slant cultures until further analysis.

qPCR analysis was performed to study the dynamics of microbial populations encoding the endo-1,4-ß-glucanase – bcsZ gene. Samples included the gut of *H. illucens* larvae grown on alternative carbon source as well on optimal diet, the enrichment substrates A1 and A2, and bulk samples (microbial cells collected from the four growth substrates). Total DNA was extracted from each sample using the FastDNA™ SPIN Kit for Soil. The qPCR was conducted using a Bio-Rad CFX96 thermocycler; data analysis was performed using Bio-Rad CFX Manager software to assess amplification efficiency and calculate relative gene expression (Figure 3).

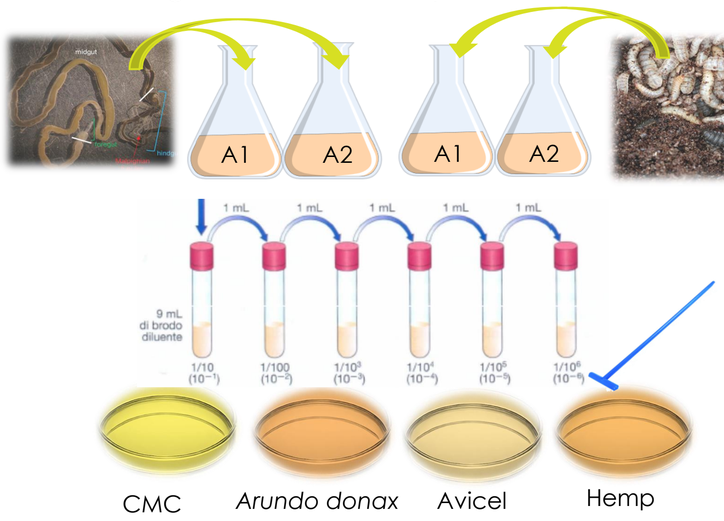

Figure 1. Inoculum of larvae grown oh hemp and gut on A1 and A2 enrichments. Serial dilution followed by the spread plate technique using four different selective substrates

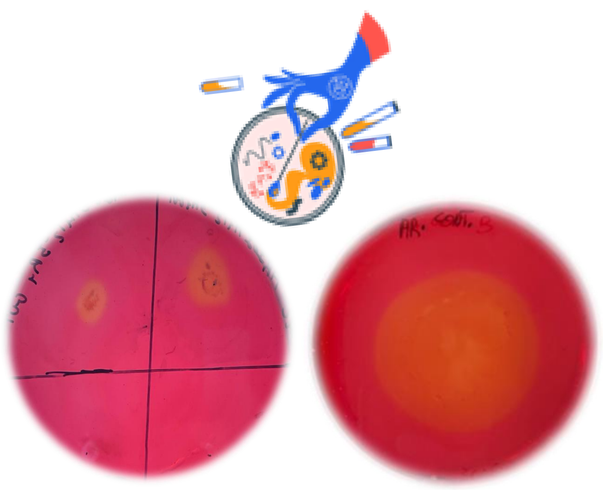

Figure 2. Semi-quantitative analysis performed by spot plating on CMC agar with 1% Congo red for detecting cellulolytic activity.

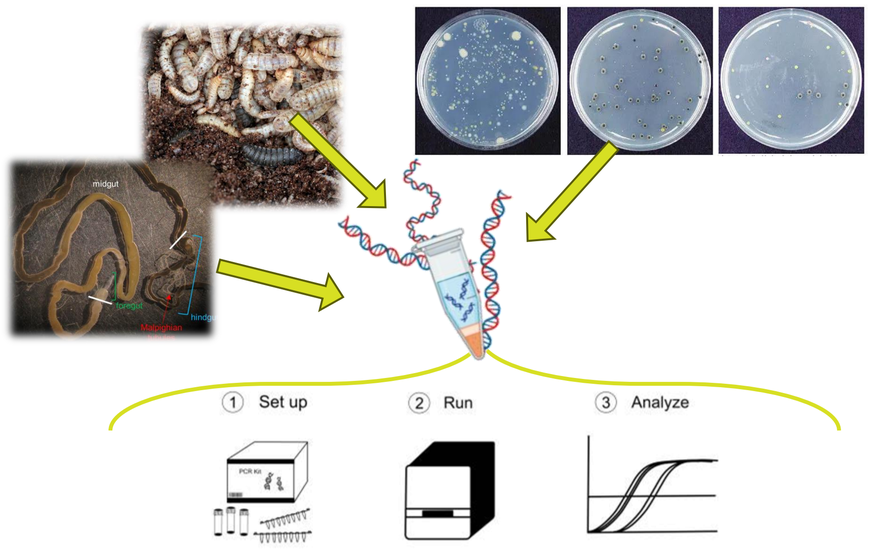

Figure 3. DNA extraction from larval biomass and gut; collection of bulk samples and DNA extraction. Quantitative PCR (qPCR) analysis steps.

Results

Inoculating two distinct samples — *H. illucens* larvae fed a hemp diet as well their corresponding gut — into two enrichment substrates (A1 and A2) yielded 546 isolates. Samples from enrichment were withdraw at three different time points (T0, T1, and T2). Specifically, 66 isolates were obtained from T0, 148 from T1, and 288 from T2, indicating a higher yield at later stages of incubation (Figure 4). From the semi-quantitative assay on CMC agar, 72 out of 436 tested isolates demonstrated cellulolytic activity, representing 16.51% of the total. Among these, five isolates produced halo zones greater than 10 mm.

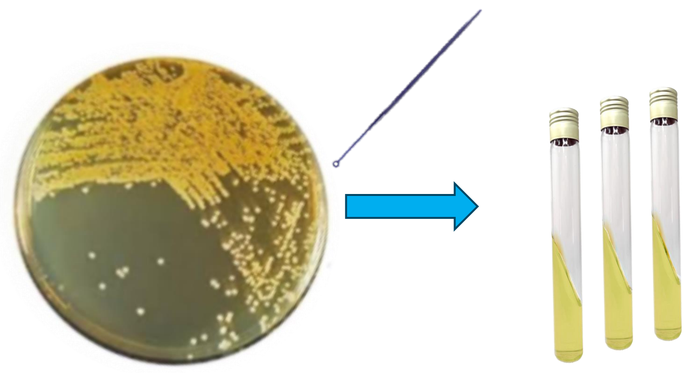

Figure 4. Purification of colonies through successive streaking and preservation of pure isolates in clarinet flasks.

The qPCR analysis of *H. illucens* samples showed that the gut samples added in the enrichment substrates A2 had greater *bcsZ* gene copies than the A1, providing insights about the potentialities of the enrichment (Figure 5 a). Otherwise also the four substrates selected for different number of gene copies; results showed that the substrates with *A. donax nor* CMC as complex carbon source combined with samples from enrichment A2 were potentially the best combo to select microorganisms with cellulolytic activity (Figure 5 b).

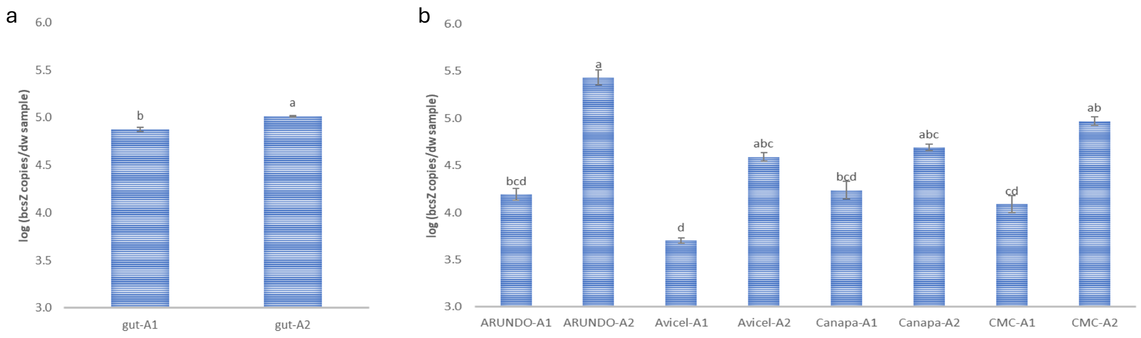

Figure 5. Bar diagrams showing *bcsZ* gene copy number of a) samples from enrichment A1 and A2, and b) bulk samples from serially diluted plates on different media. The error bars represent the means ± SD of three replicates. Different letters indicate significant differences ($P < 0.05$).

References

[1]. L. Hankin, S. Anagnostakis. Solid media containing carboxymethyl cellulose to detect CM celllulase activity of microorganisms. *J Gen Microbiol*. 1977; 98: 109-115.

[2]. V. Ventorino, A. Aliberti, V. Faraco, A. Robertiello, S. Giacobbe, D. Ercolini, A. Amore, M. Fagnano, O. Pepe. Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application. *Sci. Rep*. 2015; 5: 8161.

[3]. M.L. Rabinovich , M.S. Melnick, A.V. Bolobova. The structure and mechanism of action of cellulolytic enzymes. *Biochem Mosc*. 2002; 67: 850–871.

This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.