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Brief Report



Selective Isolation and Screening of Actinobacteria Strains Producing Lignocellulolytic Enzymes Using Olive Pomace as Substrate

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Background: Olive pomace, as the main by-product of the olive oil industry, is recently recycled as fermentation substrate for enzyme production.

Objectives: Actinobacteria isolates were separated from an Algerian soil under olive pomace cultivation and were evaluated for their lignocellulolytic enzymes production.

Materials and Methods: Isolates of Actinobacteria were separated from soils around oil mills using four isolation media, among them three were enriched by olive pomace. The isolates were screened for their cellulolytic, xylanolytic and ligninolytic activities. Isolates with potential of producing lignocellulose-degrading enzymes were selected under submerged fermentation based olive pomace.

Results: Ninety isolates of Actinobacteria were separated from soil samples. M3 medium (raw pomace autoclaved alone) was the best isolation medium (68 strains), whereas, the soil from oil mill with continuous system (S1) led to separation of 52 strains. Among the 90 isolates, 82 were shown promising enzyme activity, 19 isolates were presented the largest zone diameter (<30 mm). S1M3I and S1M3II isolates were exhibited the highest values.

Conclusions: Olive pomace with medium low cost and high titers of enzymes can be valorized by culture of Actinobacteria to produce lignocellulolytic enzymes for industrial applications.

Keywords: Actinobacteria; Lignocellulolytic enzymes; Olive pomace; Submerged fermentation

1. Background

Actinobacteria, highly abundant filamentous Gram positive bacteria are ubiquitously present in all natural substrates and soil (1-2). Actinobacteria are involved in recycling hard-to-degrade organic matter such as cellulose, cell wall matrix polysaccharides and lignin (3-4).

Use of low-cost residues from agro-industries, as substrates for growing microorganisms, may constitute an interesting alternative in enzyme industry (4). According to the National Agency for Development of Investment (ANDI, Algeria), 87500 tons of olive pomace are being produced and discarded that can act

as pollutant in near future (5). Olive pomace consists of lignocellulosic matrix with phenolic compounds, uronic acids, and oily residues and may represent an important alternative source for enzymatic processes and biofuel production (4). However, studies dealing with lignocellulolytic production by Actinobacteria using olive pomace residues are rare.

Here, Actinobacteria isolates from an Algerian soil under olive pomace cultivation were separated, and the production of carboxymethyl cellulases (CMCases), xylanases and laccases were evaluated qualitatively and quantitatively.

2. Materials and Methods

2.1. Collection of lignocellulosic Samples

Olive pomace was collected from oil mill (Algeria), dried at 22°C for 3 weeks, ground to a fine powder, passed through sieves ($\varnothing \leq 75 \mu\text{m}$ and $\varnothing \leq 1 \text{ mm}$). One portion of the sieved pomace was pretreated by hexane to remove lipids.

2.2. Soil Samples and Microorganisms

The samples were taken from the soil of natural discharge of three oil mills; S1: continuous system (annual activity), S2: traditional system (abandoned for 20 years), and S3: traditional system (annual activity). Actinobacteria from soils were isolated using standard dilution plate method (6) on four different media: M2 (Starch Casein Agar medium) containing 10 g.L⁻¹ soluble starch, 0.3 g.L⁻¹ casein, 1 g.L⁻¹ glucose, 2 g.L⁻¹ KNO₃, 2 g.L⁻¹ K₂HPO₄, 2 g.L⁻¹ NaCl, 0.05 g.L⁻¹ MgSO₄.7H₂O, 0.02 g.L⁻¹ CaCl₂, 0.01 g.L⁻¹ FeSO₄.7H₂O, 18 g.L⁻¹ agar, pH 7.2 and autoclaved at 120°C. 20 min⁻¹) (7).

M1 containing: 8 g.L⁻¹ raw pomace powder ($\varnothing=75\mu\text{m}$), 2 g.L⁻¹ soluble starch, 0.3 g.L⁻¹ casein, 1 g.L⁻¹ glucose, 2 g.L⁻¹ KNO₃, 2 g.L⁻¹ K₂HPO₄, 2 g.L⁻¹ NaCl, 0.05 g.L⁻¹ MgSO₄.7H₂O, 0.02 g.L⁻¹ CaCl₂, 0.01 g.L⁻¹ FeSO₄.7H₂O, 18 g.L⁻¹ agar, pH 7.4 and then sterilized.

M3 and M4 containing: 8 g.L⁻¹ raw pomace powder ($\varnothing=75 \mu\text{m}$), and 8 g.L⁻¹ pretreated pomace powder ($\varnothing=75 \mu\text{m}$), respectively (Sterilized alone), and aqueous phase containing: 2 g.L⁻¹ soluble starch, 0.3 g.L⁻¹ casein, 1 g.L⁻¹ glucose, 2 g.L⁻¹ KNO₃, 2 g.L⁻¹ K₂HPO₄, 2 g.L⁻¹ NaCl, 0.05 g.L⁻¹ MgSO₄.7H₂O, 0.02 g.L⁻¹ CaCl₂, 0.01 g.L⁻¹ FeSO₄.7H₂O, 18 g.L⁻¹ agar, pH 7.8 and then sterilized. Rifampicin (2.5 mg.mL⁻¹) and amphotericin B (75 mg.mL⁻¹) were added to all media in order to inhibit bacterial and fungal contaminations, respectively (6). Plates were incubated at 28°C for 7d. Colonies showing an Actinobacteria-like appearance were purified and selected based on the Gram staining.

2.3. Screening of CMCCase, Xylanase and Laccase Producers

A preliminary qualitative analysis was conducted by carboxymethyl cellulose (CMC) and xylan containing agar plates method described by Boroujeni *et al.* (1) and guaiacol containing agar plates method described by Lu *et al.* (8).

2.4. Submerged Fermentation

Submerged fermentation was carried out in Erlenmeyer flasks (250 mL) containing 50 mL of the production medium containing: 30 g.L⁻¹ raw olive pomace powder

($\varnothing \leq 1 \text{ mm}$), 3 g.L⁻¹ (NH₄)₂SO₄, 1.2 g.L⁻¹ NaNO₃, 1.5 g.L⁻¹ KH₂PO₄, 3 g.L⁻¹ K₂HPO₄, 0.2 g.L⁻¹ MgSO₄.7H₂O, 0.05 g.L⁻¹ CaCl₂, 0.01 g.L⁻¹ MnSO₄.7H₂O, 0.001 g.L⁻¹ FeSO₄.7H₂O, 0.05 g.L⁻¹ yeast extract, pH 7.4. The flasks were autoclaved (olive pomace alone), inoculated with three agar disks ($\varnothing=6 \text{ mm}$) taken from the 7 d old stock culture and incubated at 30°C for 6 d on a rotary shaker at 150 rpm. The crude enzyme preparations were obtained by centrifugation (12,857 ×g for 20 min) and used for enzyme assay.

2.5. Measurement of CMCCase, Xylanase and Laccase Activities

Xylanase and CMCCase activities were determined by measuring the release of reducing sugar according to Tuncer *et al.* (9). Laccase activity was assayed according to Criquet *et al.* (10). The results were expressed in units (U), where U is defined as the amount of enzyme required to liberate 1 μM of xylose, glucose and quinone per min.

2.6. Statistical Treatment

One-way analysis of variance (ANOVA) was used to analyze data and multiple pair-wise comparisons were performed by the Tukey test using Xlstat® software.

3. Results

After processing of soil samples, a total of 90 Actinobacteria strains were isolated. Most of the isolates were separated on M3 medium (68 isolates) and negative results belong to M1 (Fig. 1). The S1 soil gave 52 isolates (Fig. 1), higher than S2 and S3 (the least number of isolates).

Among the 90 isolates, 82 showed promising enzyme activities (CMCase, xylanase and laccase) with diameter zones varying between 19 mm and 53 mm (Table 1). Nineteen isolates (1), with diameters greater than 30mm, were the most aggressive types.

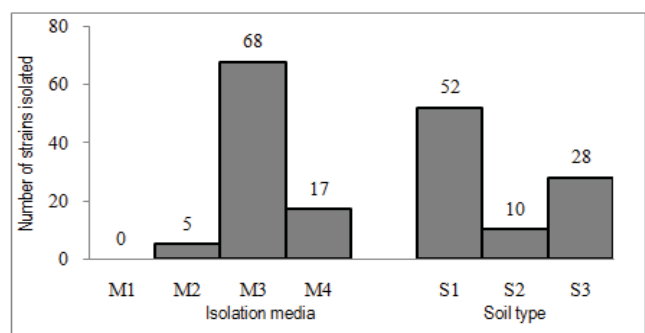


Figure 1. Number of Actinobacteria isolated from different soils (S1, S2 and S3) and on different media (M1, M2, M3 and M4).

Table 1. Screening for lignocellulolytic enzymes produced by different Actinobacteria isolates.

Number of isolates	CMCase	Xylanase	Laccase
8	-	-	-
11	+	-	-
19	-	+	-
13	+	+	-
20	++	-	-
16	++	+	-
2	+++	++	Pre.
1	+++	+++	Pre.

Pre.: Presence of activity - : No activity, + <30 mm, ++ (30-40 mm) and +++ >40 mm.

The isolates were further characterized by carrying out enzymatic assays using CMC and xylan as substrates. Furthermore, 3 isolates giving promising enzymatic activities were tested using syringaldazine. All tested isolates exhibited activities, comprised between 1.44-0.31 U.mL⁻¹ for CMCase, 6.65-0.79 U.mL⁻¹ for xylanase and 5.63 x 10⁻³-2.15 x 10⁻³ U.mL⁻¹ for laccase (Table 2; p <0.05). The best results were obtained with S1M3I and S1M3II strains for all enzyme activities studied.

4. Discussion

Actinobacteria isolates were separated from soil around olive oil mill. It was expected that due to the presence of decomposing olive pomace, the main bacterial species have the capability of producing enzyme that were able to degrade lignocelluloses. The higher number of isolates obtained on M3 medium compared to M2 and M4 can be explained by the fact that the isolation medium has the same characters as the natural environment of sampling. Indeed, Vance and Chapin (11) reported that microbial growth is influenced by the characteristics of its natural environment, that depends upon the chemistry of the organic matter, soil moisture, soil temperature and physical access of enzyme(s) to substrate(s).

Autoclaving of olive pomace, in aqueous medium (the case of M1 medium) induces extraction and diffusion of phenolic compounds in the medium, causing modifications of its physical and chemical characteristics. An increase in temperature increases the efficiency of the extraction since heat render the cell walls permeable (12) and decreases the viscosity of the solvent (13). On the other hand, several studies have demonstrated that polyphenols are responsible for phytotoxic and antimicrobial actions (12,14).

The difference obtained between the two soils from traditional oil mills (S2 and S3) is due to the time given

to the natural fauna from soil to degrade the olive pomace (15) and induction of microflora formation (16), among other Actinobacteria. According to Kuzyakov *et al.* (17) very long periods would be necessary to obtain measurable soil transformations. Soils with long-term exposure to contamination with organic compounds have been shown to have structural and functional microbial communities with the ability to adapt and grow under these conditions (2). Indeed, the oil mills with continuous system produced exhausted pomace, unlike to the traditional ones, so less toxic compound (polyphenol) were formed and fatty acids were released by the degradation of lipids in the soil (5). The lipid hydrolysis releases acids, which causes a decrease in soil pH (13). Lipids, organic acids and mostly phenols are considered responsible for the phytotoxicity, representing a severe risk of water and soil pollution (2).

Table 2. Lignocellulolytic enzyme activities of the selected Actinobacteria strains.

Isolates	CMCase activity (U.mL ⁻¹)	Xylanase activity (U.mL ⁻¹)	Laccase activity (10 ⁻³ U.mL ⁻¹)
S1M3I	1.44 ± 0.01 ^a	6.65 ± 0.16 ^a	3.42 ± 0.18 ^b
S1M3II	1.42 ± 0.04 ^a	5.40 ± 0.42 ^b	5.63 ± 0.38 ^a
S1M3III	0.57 ± 0.00 [#]	2.97 ± 0.10 ^c	2.15 ± 0.14 ^c
S3M4I	0.67 ± 0.04 ^f	1.82 ± 0.75 ^d	-
S3M3I	0.95 ± 0.04 ^c	2.08 ± 0.03 ^d	-
S1M3IV	0.89 ± 0.04 ^{d,e}	1.79 ± 0.05 ^d	-
S1M3V	1.06 ± 0.02 ^b	0.84 ± 0.06 ^h	-
S1M3VI	0.80 ± 0.05 ^e	1.17 ± 0.12 ^f	-
S3M3II	0.66 ± 0.04 ^f	1.16 ± 0.04 ^f	-
S3M3III	0.56 ± 0.01 ^{g,h}	1.21 ± 0.06 ^{e,f}	-
S1M3VII	0.53 ± 0.02 ^h	1.12 ± 0.07 ^g	-
S3M3IV	0.50 ± 0.01 ^h	0.79 ± 0.003 ^h	-
S1M3VIII	0.68 ± 0.02 ^f	0.80 ± 0.02 ^h	-
S3M2I	0.53 ± 0.08 ^h	0.93 ± 0.01 ^h	-
S3M3V	0.58 ± 0.04 ^{g,h}	1.40 ± 0.06 ^e	-
S3M3VI	0.94 ± 0.01 ^{c,d}	1.06 ± 0.04 ^{g,h}	-
S3M3VII	0.31 ± 0.03 ⁱ	0.97 ± 0.09 ^h	-
S3M4II	1.03 ± 0.02 ^{b,c}	1.16 ± 0.06 ^{f,g}	-
S2M4I	0.56 ± 0.03 ^g	1.61 ± 0.04 ^{d,e}	-

- No measured

Different letters in same column indicate significant difference (p <0.05). Results are ranked in ascending order: a>b>c>d>e>f>g>h>i.

The production of enzymes was tested by fermentation in a submerged medium using olive pomace. At the present time, there is no citation in the scientific literature describing the use of olive pomace for the production of hydrolases (CMCases and xylanases) and oxidases (laccase) enzymes by the Actinobacteria. Grigorevski *et al.* (18) reported CMCCase activity of 0.15 U.mL⁻¹ by cultivation of *Streptomyces drozdowiczii* on corn steep liquor. Sharma and Bajaj (19) reported xylanase activity of 2.21 U.mL⁻¹ by cultivation of *Streptomyces* sp. CD3 on wheat bran. Laccase activity was mainly reported for fungi. Lakhtar *et al.* (20) reported laccase activity of 0.2 U.mL⁻¹ by cultivation of *Lentinula edodes* on olive mill waste water.

The results obtained suggest that Actinobacteria are a good producer of cell-wall degrading enzymes using olive pomace as a carbon source which can be valorized by submerged culture. Considering the ratio between the high titers of enzymes produced and the low olive pomace cost and the fact that is a very abundant agricultural residue in Algeria.

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