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Research Article

Isolation and Screening of Fungal Culture Isolated From Algerian Soil for the Production of Cellulase and Xylanase

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ABSTRACT

Lignocellulolytic enzymes constitute a very large group of extracellular proteins secreting by fungi who is ecologically involved in the degradation of a variety of complex materials, a property that is attributed to a battery of enzymes produced by these microorganisms like cellulases and xylanases who are of significant industrial value and relevance. Forty fungal isolated from rich soil in organic matter were screened for lignocellulolytic enzymes production, its organized on the basis of their hydrolytic potential of cellulose and xylan. The isolates strains presented enzymatic activity which was ranked as follows: cellulolytic (56%), xylanolytic (44%). Some selected strains that produce high levels of enzymes (cellulase, xylanase) grown in submerged fermentation (SmF) and were quantitatively evaluated. The fermentation experiments were carried out in shake flasks. The highest CMCase (5,10 IU/ml) and xylanase (98,25 IU/ml) activities were obtained from *Trichoderma* sp strain Mtr6 isolate.

Keywords: Fungi, *Trichoderma* sp, lignocellulolytic enzymes, soil, screening, organic matter.

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INTRODUCTION

Lignocellulose biomass is abundant in nature, and represents more than half of the organic matter produced globally via plant photosynthesis. Bioconversion of relatively inexpensive lignocellulosic biomass into biofuels and value-added products can effectively alleviate pressure of energy supply and benefit sustainable development¹. Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as biomass degraders for large-scale applications due to their ability to produce large amounts of extracellular lignocellulosic enzymes². Enzymatic hydrolysis is an important technical, but expensive step in the process to obtain enzyme derived products. Thus, the production of efficient enzymes is of great interest for this biotechnological application³. Indeed, for the production of industrially important enzymes, such as different proteases, carbohydrases and lipases⁴; fungi isolated from soil are known to be potential candidates⁵. According to Tasia and Melliawati⁶, microbial proteins are often more stable than those from other source, such as animal or plant origins, for

this reason the industrial enzymes market prefers microbial enzymes⁷.

Microorganisms can be easily cultured in large quantities in a very short period of time. Fungi in particular are preferred for enzyme production, since they grow easily in a diversity of substrates and the large-scale production of enzyme is a relatively easy process in biotechnological industries. Therefore, commercial enzymes that are on the market are often of fungal origin, including cellulases and xylanase⁸. These two groups of enzymes have numerous application and massive biotechnological potentials for the various industrial sectors, including chemical, food, brewer and wine, animal feed, textile and laundry, pulp, paper and agriculture^{8,9,10,11} and are currently being used significantly in the commercial bioconversion of lignocellulosic biomass to bioethanol¹².

Submerged fermentation (SmF) has been traditionally used for the production of industrially important enzymes because of the ease of handling and greater control of environmental factors such as temperature and pH¹³. Many

researchers studied about extracellular enzyme by microorganisms from many sources^{14,15,16,17,18,19}. The aim of this study was to select fungi from soil or decomposing organic material for the production of two industrially important extracellular lignocellulolytic enzymes: cellulase and xylanase using solid media screening and liquid media assays, as well as to access the enzymatic activity of most promising isolates.

MATERIALS AND METHODS

Isolation of fungi from soil

The fungi used in this study were collected from soil samples of rich organic matter from several regions of Algeria (Mascara from the West, and Bordj Bou Arrerridj from the East) within a depth of 5 to 10 cm after removal of superficial layer. Serial dilution method was carried out for isolation of pure culture. The isolates were further inoculated on sterile PDA (potato dextrose agar) plates and incubated at 28 °C for 3-5 days. These fungi were then subcultured and preserved in pure form. Colonial morphology and microscopic examinations of the various isolates of pure cultures were used to determine the reproductive and vegetative structures²⁰.

Screening of fungal for lignocellulolytic enzymes production

First screening was done by qualitative method (agar plate method, hydrolysis zone) and second screening method (liquid culture). Lignocellulolytic enzyme activity was tested on solid media for all the isolated cultures. Strains whose result positive in first screening were tested on liquid media (quantitative screening).

Enzymatic assays in solid media (first screening)

Enzymes production is sought qualitatively on solid medium. Fungal strains were screened for cellulase and xylanase. Solid culture media containing targeted substrates as a single source of carbon and energy; 1% for each substrate used and 1.8% (v/w) agar.

Cellulolytic and xylanolytic activities: The culture medium described by Mandes and Weber²¹ was used to study the ability of the fungal isolates to produce cellulase and xylanase using 1% of carboxymethyl cellulose (CMC) and birchwood respectively. When the colony diameter was approximately 30 mm, the plates were flooded with 0.25% w/v aqueous iodine (I₂ and KI) and left for 15 minutes, then poured off the staining material and washed the agar surface with distilled water. Then, the plates were flooded with 1M NaCl to destain for 5 minutes and then the destaining fluid was poured off. The hydrolysis zone of cellulose appeared as a clear zone around the colony¹⁹. Xylan degradation around the colonies appeared as a yellow-opaque area against a blue/reddish purple color for undegraded xylan²².

Media were autoclaved and inoculated with agar blocks (6 mm in diameter) from one-week old fungal colony grown on PDA plates in the center of the medium's plates. The plates were incubated at 28 °C for 5 or 7 days. After incubation, activities were revealed by the appearance of a ring-shaped clear zone surrounding colony growth.

Production of lignocellulolytic enzyme using liquid culture (quantitative screening)

The production of hydrolytic enzymes (cellulase and xylanase) is carried out in 250ml Erlenmeyer flasks, containing 50ml of the liquid culture medium. To obtain hydrolytic enzymes the fungi were grown in this medium with 1% of substrate (wheat bran) for cellulase and xylanase activity, and then sterilized by autoclaving at 121°C for 15 min before inoculation. Inoculum in the form of mycelia disc is prepared by cutting the agar plug from the periphery of the actively grown fungi²³.

Mycelium disks (6mm in diameter) were excised from PDA plates and used to inoculate the contents of Erlenmeyer flasks. The culture was incubated in shaking incubator for 7 days at 28 °C, after incubation it was filtered using Whatman no.1 filter paper, the filtrates were centrifuged at 10000 g for 15 min at 4°C. The clear supernatants were used as the crude extracellular enzyme's sources²⁴.

Determination of enzymes activities

Cellulase and xylanase activities

According to Ghose (1987)²⁵, endoglucanase and xylanase activities were determined using DNS (3,5-dinitro salicylic acid) using carboxymethyl cellulose and birchwood xylan at (2%) as substrates in sodium citrate buffer (50 mM, pH 4.8). The reaction mixture contained 0.5 ml of culture filtrate and 0.5 ml substrate was incubated at 50°C for 30 min for enzymatic reaction.

The liberated reducing sugars (glucose/ xylose) were measured by 3,5-dinitrosalicylic acid (DNS) method of Miller (1959)²⁶. Absorbance of the solution was measured at 540 nm using UV-VIS spectrophotometer. One international unit was defined as the amount of enzyme which liberates 1μmol of reducing sugar per milliliter per minute under the given assay conditions.

Statistical methods

Data obtained were statistically analyzed using variance analysis of (ANOVA) Microsoft EXCEL 2016.

RESULT AND DISCUSSION

According to Jahangeer et al (2005)¹⁵ fungi are well-known as agents of decomposition of organic matter in general and lignocellulosic substrate in particular by secreting cellulases and xylanases who are a key enzyme that can be effectively used to solve challenges associated with energy inadequacy and environmental pollution²⁷.

Qualitative tests are powerful tools and particularly useful in screening large numbers of fungal isolates for several classes of enzyme²².

The fourty fungal isolates used in qualitative tests were isolated from rich soil in organic matter and were submitted to the hydrolytic tests to confirm the production of cellulase and xylanase. The results for the qualitative assays performed on solid media for the production of hydrolytic enzymes by fungi are presented in figure 1 and table 1.

Figure 1: Detection of the enzyme activities using Petri dishes containing specific mediums. (A) Cellulolytic activity, (B) Xylanolytic activity.

Table 1: Enzyme activity of fungal isolates on agar plates

STRAIN	Cellulase	Xylanase
TRC1	-	-
TRC2	++	++
TRC3	-	-
TRC4	+++	+++
TRC5	++	++
TRC6	+	++
TRC7	-	-
TRC8	+	+
TRC9	+	+
TRC10	+	+
TRC11	++	++
TRC12	-	-
TRC13	-	-
TRC14	-	-
TRC15	-	-
TRC16	+++	+++
ALT1	+++	+/-
ALT2	++	+
ALT3	-	-
ALT4	+	-
ALT5	+++	+++
FUS1	-	-
FUS2	-	-
VOB1	+++	+++
CLD1	+++	+
CUV1	+	+
CUV2	+	-
PNC1	+++	+++
PNC2	++	+/-
PNC3	++	+
APS1	+++	+++
APS2	+++	+++
APS3	+++	+++
APS4	-	-
APS5	-	-
APS6	-	+/-
APS7	-	-
APS8	-	+/-
APS9	-	-
APS10	-	-

+++ Strong activity, ++ moderate activity, + Low activity, +/- suspicious activity, - no hydrolysis zone

Among the 40 strains, 23 strains showed a cellulase activity, 18 are capable to produce xylanase. The perceptual distribution of the hydrolytic enzyme production among the 40 tested fungi was presented in figure 2.

Figure 2: Percentage of fungal isolates according to its hydrolytic activities.

The figure 2 confirms that from 40 strains isolated, 56% presented cellulolytic activity. Of these, isolates TRC4 (*Trichoderma* sp), TRC16 (*Trichoderma* sp), ALT1 (*Alternaria* sp), ALT5 (*Alternaria* sp), VOB1 (*Beauveria* sp), CLD1 (*Cladosporium* sp), PNC1 (*Penicillium* sp), APS1 (*Aspergillus* sp), APS2 (*Aspergillus* sp) and APS3 (*Aspergillus* sp) presented a larger hydrolysis zone, which suggest that these fungi have higher cellulase activity than the other isolates. TRC2 (*Trichoderma* sp), TRC5 (*Trichoderma* sp), TRC11 (*Trichoderma* sp), ALT2 (*Alternaria* sp), PNC3 (*Penicillium* sp), PNC2 (*Penicillium* sp) have moderate activity and TRC8 (*Trichoderma* sp), TRC9 (*Trichoderma* sp), TRC10 (*Trichoderma* sp), TRC6 (*Trichoderma* sp), ALT4 (*Alternaria* sp), CUV1 (*Culvularia* sp), CUV2 (*Culvularia* sp) with low activity.

For the xylanase production, 44% of the isolates showed positive results: TRC4 (*Trichoderma* sp), ALT5 (*Alternaria* sp), VOB1 (*Beauveria* sp), PNC1 (*Penicillium* sp), APS1 (*Aspergillus* sp), APS2 (*Aspergillus* sp), APS3 (*Aspergillus* sp) presented larger halos in the solid media, indicating higher xylanolytic activity of these isolates. While TRC2 (*Trichoderma* sp), TRC5 (*Trichoderma* sp), TRC6 (*Trichoderma* sp), TRC11 (*Trichoderma* sp) have moderate activity and CLD11 (*Cladosporium* sp), ALT2 (*Alternaria* sp), CUV1 (*Culvularia* sp), PNC3 (*Penicillium* sp), TRC8 (*Trichoderma* sp), TRC9 (*Trichoderma* sp), TRC10 (*Trichoderma* sp) with low activity.

For quantitative estimation, *Trichoderma* sp (TRC6) produced maximum cellulase (5,10 UI/ml) and xylanase with (98,25UI/ML) although she is not among the strains that presented larger hydrolysis zone. Xylanase activity is high compared to cellulase because hemicelluloses are the most easily polysaccharide compounds hydrolyzed of the plant cell wall²⁸.

The cellulases term refer to a group of enzymes that catalyze the hydrolysis of cellulose into sugars. Cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, the most abundant carbohydrate produced by plants²⁹. Cellulases are widely used such as in the food, fuel, textiles, pulp and paper industries^{30,31}.

According to Girio *et al* (2010)³²; Selvam *et al* (2014)³³; Kandasamy (2016)³⁴ xylans are the predominant compounds in the hemicellulose fraction, it is depolymerized to xylose and other sugars by xylanases (E.C. 3.2.1.8) who are

hydrolytic enzymes with wide applications in several industries, such as biofuels, paper, deinking, food and feed³⁵.

Fungi like *Trichoderma* sp secrete a large number and a variety of enzymes that can act the polycassharides found in plant cell walls. These enzymes include cellulases, hemicellulases, pectinases, esterases, oxidoreductases and proteases³⁶. Xylanases are among the most known enzymes; therefore, this fungal genus is suited for further examination of function and application of these enzymes³⁷.

Trichoderma reesei Rut C-30 is the most well-known *Trichoderma* strain producing several xylanases and cellulases with different biochemical properties and specificities for substrates, as predicted by genome sequence³⁸, and also many enzyme preparations obtained from the large-scale cultivation of this fungus have been commercialized. Xylanases from other *Trichoderma* species have also been studied as those from *Trichoderma harzianum*, *Trichoderma lignorum*, *Trichoderma longibrachiatum*, *Trichoderma koningii*, *Trichoderma pseudokoningii* and *Trichoderma viride*^{39,40}.

The polysaccharides especially celluloses and hemicelluloses are very cheap and easily available as wastes from industries, like paper and pulp, agriculture, food and feed and municipal. In developing countries these wastes are not been discarded or treated properly and become the major cause of environmental pollution².

Nineteen strains; TRC2 (*Trichoderma* sp), TRC4 (*Trichoderma* sp), TRC5 (*Trichoderma* sp), TRC6 (*Trichoderma* sp), TRC8 (*Trichoderma* sp), TRC9 (*Trichoderma* sp), TRC10 (*Trichoderma* sp), TRC11 (*Trichoderma* sp), TRC16 (*Trichoderma* sp), ALT1 (*Alternaria* sp), ALT2 (*Alternaria* sp), ALT4 (*Alternaria* sp), ALT5 (*Alternaria* sp), VOB1 (*Beauveria* sp), CLD1 (*Cladosporium* sp), CUV1 (*Culvularia* sp), CUV2 (*Culvularia* sp), PNC1 (*Penicillium* sp), PNC2 (*Penicillium* sp), PNC3 (*Penicillium* sp), APS1 (*Aspergillus* sp), APS2 (*Aspergillus* sp), APS3 (*Aspergillus* sp) produced the two lignocellulolytic enzymes (cellulase and xylanase).

Fifteen isolates: TRC1 (*Trichoderma* sp), TRC3 (*Trichoderma* sp), TRC7 (*Trichoderma* sp), TRC12 (*Trichoderma* sp), TRC13 (*Trichoderma* sp), TRC14 (*Trichoderma* sp), TRC15 (*Trichoderma* sp), ALT3 (*Alternaria* sp), FUS1 (*Fusarium* sp), FUS2 (*Fusarium* sp), APS4 (*Aspergillus* sp), APS5 (*Aspergillus* sp), APS7 (*Aspergillus* sp), APS9 (*Aspergillus* sp), APS10

(*Aspergillus* sp) were non producers of any of the lignocellulolytic enzymes studied. However, a negative result does not confirm the inability of a strain to produce the enzyme. This mean that the medium is inadequate for the detection of the enzyme, or the enzyme has not been released from the mycelium⁴¹.

Fungi in general are well characterized microorganisms due to their capacity to produce a wide range of enzymes to digest complex materials in the environment into solubilized breakdown products that can be up taken into the hyphae and used as nutrients for surviving⁴².

Figure 3. The lignocellulolytic enzymes activities of fungal isolates.

A: Cellulolytic activity, B: Xylanolytic activity.

CONCLUSION

In present study, forty different fungal isolates from rich soil in organic matter were screened for the presence of lignocellulolytic cextracellular enzymes such as cellulase and xylanase which has grown on specific mediums (qualitative activity) and under submerged fermentation (quantitative activity). The order of enzymes activities found in this study with percentage for the isolated microorganisms are cellulolytic then xylanolytic. For cellulase and xylanase activities, the highest producing strain was *Trichoderma* sp. Based on these results; this strain has a major role in the degradation of organic matter.

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