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A Combination of Solid Mandels Medium, CMC, and Congo Red Technique for Rapid, Sensitive and Reproducible Screening of Cellulase-Producing Fungi

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Abstract

The bioconversion of lignocellulosic biomass into monomeric sugars, by the action of cellulase enzymes, is the main economic problem hindering the profitable use of this abundant source of energy. Trichoderma genus is important biotechnologically due to their ability to produce a wide spectrum of cellulase enzymes and bioactive compounds. There are different techniques to detected qualitative and quantitative cellulase enzymatic secretion of fungal isolates. In this work, we have analyzed the cellulase secretion from four isolates of Trichoderma genus by qualitative and quantitative assays, with the aim to detect a rapid, sensitive and reproducible way for screening cellulase-producing fungi. Two solid medium in three conditions of pH: 3.5, 4.5 and 5.5 and two incubation time were assayed to evaluate the qualitative enzymatic secretion of fungal isolates. The synergic effect of cellulase complex was quantitatively determined by filter paper activity. The correlation between qualitative screening with Congo red technique and quantitative screening with the DNS reagent method had already been reported, but not reported the composition of the medium to use for it. In this study, we could standardize a solid medium to detect qualitative cellulase activities with Mandels medium as a nitrogen complex at pH 4, CMC as only carbon source and Congo red technique. This method was rapid, sensitive and reproducible way for screening cellulase-producing fungi.

Keywords: Cellulase - qualitative and quantitative assays – Mandels – CMC – FPA – Trichoderma - Misiones, Argentina

Abbreviations : 2G: Second Generation; CMC: Carboxymethyl Cellulose; FPA: Filter Paper Assay; IUPAC: International Union of Pure and Applied Chemistry tests; DNS: Dinitrosalicyclic acid; EGs: endo-1,4-β-glucanases; CBHs: Cellobiohydrolases; BGLs: β-glucosidases; LBM: Laboratorio de Biotecnologia Molecular

Introduction

The availability of fossil fuel resources and the increasing energy demand are the main driving forces in the search for alternative energy sources. The large-scale replacement of petroleum fuels by biofuels, such as bioethanol from lignocellulosic materials (bioethanol 2G) appears to be a powerful approach to solve the growing energy demands [1]. Bioethanol 2G is particularly promising because it can use the capabilities of biotechnology to reduce production costs, employ abundant and low cost raw materials, has a higher octane rating and is an environmentally clean product [2].

Lignocellulose biomass is abundant in nature and represents more than half of the organic matter produced globally via plant photosynthesis [3]. Cellulose, a type of homogeneous polysaccharide that exists as units of cellobiose connected by β-1,4-glycosidic bonds, is the most abundant renewable biomass in nature [4, 5]. Bioethanol 2G production from lignocellulosic materials involves three

main steps:

1. Pretreatment,

2. Enzymatic hydrolysis of cellulose and hemicellulose to glucose, and

3. Ethanol fermentation [6,7].

Hydrolysis of cellulose to glucose requires the action of the cellulase complex, composed by three groups of enzymes: endo-1,4-β-glucanases (EGs - EC 3.2.1.4) randomly cut β-1,4-bonds of cellulose chains generating new ends; cellobiohydrolases (CBHs - EC 3.2.1.91) act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains liberating either cellobiose or soluble cellodextrins as major products; and β-glucosidases (BGLs - EC 3.2.1.21) hydrolyze soluble cellodextrins and cellobiose to glucose [5,8,9].

In recent years the interest in cellulase has increased due to many potential applications for these types of enzymes. The production of cellulase is a key factor in the hydrolysis of cellulosic material and it is essential to make the process economically viable [8,10,11].

Many filamentous fungi are widely used for producing cellulolytic enzymes to degrade lignocellulosic biomass [3]. Trichoderma genus [12] has been extensively studied related to its high ability of secreting cellulose-degrading enzymes. This genus comprises a large number of saprotrophic species with a worldwide distribution [13]. Members of this genus are important biotechnologically due to their ability to produce a wide spectrum of cellulase enzymes and bioactive compounds [14-18]. Therefore many efforts have been made in obtaining new microorganisms of this genus to produce cellulase enzymes with higher specific activity and outstanding efficiency [8,19,20]. The forest in Misiones (Argentina) has a very rich biodiversity for searching new fungal microorganisms. However, studies on cellulase producing fungal isolates from Misiones and Argentina remain very limited.

There are different techniques to detected qualitative and quantitative cellulase enzymatic secretion of fungal isolates. Currently, filter paper assay (FPA) it is used to measure the hydrolytic potential of cellulase enzyme mixtures from some microorganisms that are used to hydrolyze a range of cellulosic substrates [21]. But the heterogeneity of insoluble cellulose, complicated synergy/competition among endoglucanase and cellobiohydrolase, and changes in ratio of enzyme/substrate pose formidable challenges in developing cellulase activity assays [22]. In this work, we have analyzed the cellulase secretion from four isolates of Trichoderma genus by qualitative and quantitative assays, with the aim to detect a rapid, sensitive and reproducible way for screening cellulase-producing fungi.

Materials and Methods

Microorganisms

Four Trichoderma isolates collected from natural ecosystem of Misiones were identified at the genus level by conventional macro-micro morphological techniques [16,23-25]. The isolates were coded as: LBM092, LBM097, LBM102 and LBM103. All these isolates were deposited in culture collection of the Universidad Nacional de Misiones (Argentina).

Culture Conditions

All the isolates were reactivated in potato-dextrose agar plates 3.9% (w/v) (PDA – Britania SA) for 5-7 days at 28 ± 1°C under constant photoperiod (24 h light). To prepare the fungal inocula for qualitative assays, 10 mm2-agar plugs from each fungal isolate grown in PDA were cut and transferred to agar plates with two different solid medium. Two solid medium were evaluated in three conditions of pH: 3.5, 4.5 and 5.5 and two incubation times to evaluate the qualitative enzymatic secretion of fungal isolates. One of them was solid Mandels' medium [17] and the other was solid Czapeck medium, with the following modifications 1.7% (w/v) agar-agar and 0.5% (w/v) sodium carboxymethyl cellulose (CMC) as only carbon source. The agar plates were incubated for 5 and 7 days at 28 ± 1°C under constant photoperiod (24 h light).

To prepare the fungal inocula for quantitative assays, spore suspensions with 107 spores/mL concentration were used as initial inoculum for each experiment and transferred to 250 mL-Erlenmeyer flasks containing 50 mL of liquid Mandels' medium [17] with 0.5% (w/v) CMC as only carbon source. The Erlenmeyer flasks were incubated in static conditions for 5 days at 28 ± 1°C under constant photoperiod [26]. Daily 1.5 mL of supernatant was taken and used as crude enzyme extract to assay extracellular cellulase secretion in quantitative screening assays.

Biochemical Analyses

Qualitative Cellulase Activities Screening Assays of Trichoderma Isolates

The qualitative cellulase activity of fungal isolates was determined by their ability to grow and form cleared zones around colonies on solid medium. The surface of the media containing the developed fungal colonies was flooded with 0.1% (w/v) Congo red solution (BioPack SA) and incubated for 15 min at room temperature. The dye was removed with sterile distilled water followed by incubation for 10 more

minutes at room temperature. Then the plates were further treated by flooding with 1M NaCl for 5 min. The ratio of the diameter of the clear zone to the diameter of the colony was measured and a scale of qualitative activity was generated [14,27,28].

Quantitative Cellulase Activities Screening Assays Of Trichoderma Isolates

The synergic effect of cellulase complex was determined by the filter paper activity assay (FPA). All the isolates were grown in liquid Mandels' medium [17]. The filter paper assay (FPA) was determined according to International Union of Pure and Applied Chemistry tests (IUPAC) [29]. FPA was assayed by measuring the release of reducing sugars in a reaction mixture containing 0.1 mL of crude enzyme, 10 mg of Whatman No. 1 filter paper as substrate and 0.2 mL of 50 mM sodium acetate buffer (pH 4.8) at 50 °C for 60 min. Reducing sugars were assayed by dinitrosalicyclic acid (DNS) method [30]. One unit of FPA activity was defined as the amount of enzyme required to liberate 1 µmol of glucose per minute from the particular substrate under the assay conditions.

Data Analysis

All experiments were conducted in triplicates. The experimental runs were designed and the results were analyzed using the Statgraphic Centurion program (StatPoint, Inc., version 15.2.05) and GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Analysis of variance was used for data analysis. The Least Significant Difference test was performed to establish differences among levels of a factor. A confidence level of 95% was applied.

Results and Discussion

Qualitative Cellulase Activities Screening Assays Of Trichoderma Isolates

The solid Mandels' medium [17] as a nitrogen complex at pH 3.5 allowed a visible and rapid enzymatic detection, with great fungal growth and sporulation. However, this acid pH interfered in the polymerization of the medium. This effect being more evident in the solid Czapeck medium, where polymerization was not directly verified (Figure 1). So, we recommend to use the solid Mandels' medium [17] as the nitrogen complex at pH 4 for the qualitative cellulase activity screening assays. No differences were observed related to the two incubation times (unpublished data), therefore the decision was made to continue working with 5 days because an appropriate screening method must be carried out in a short time [31].

Many authors reported that the Mandels' medium is a complex nitrogenous source that induces cellulase secretion

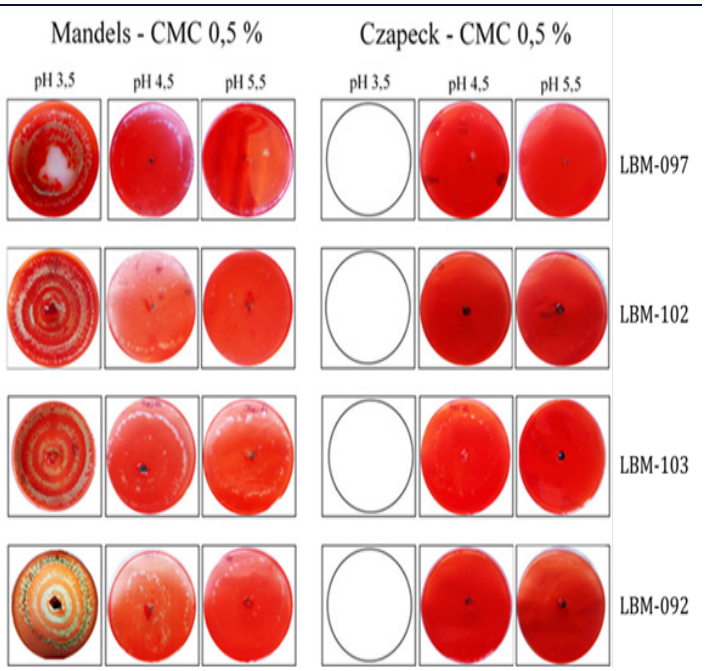

Figure 1: Standardization of solid medium to evaluate Qualitative cellulase activities screening assays of Trichoderma isolates. Both solid medium were detected with 0.1% (w/v) Congo red solution (BioPack SA) and incubated for 15 min at room temperature. The empty circle indicates that the culture medium did not solidify.

in microorganisms [17,26,32-34]. CMC substrate is a water-soluble cellulose derivative and is a useful substrate for detection of cellulase production because it is degraded quickly by microorganisms [14,17,26,31,35-37]. The Congo red dye was used as an indicator for β-1,4-glycosidic bonds degradation in an agar medium. This simple diffusion technique provides a rapid and sensitive screening test for cellulolytic microorganisms [14,31,38].

This method in plates resulted simple, rapid and well adapted for screening of a large number of samples of the same genus. Likewise, Hankin & Anagnostakis [39], Doolotkeldieva & Bobusheva [14] and Florencio et al. [38] reported that the extracellular enzymes can be produced in liquid or solid media, although the use of solid media enables rapid assays and can be useful for the isolation of cellulase-producing organisms from natural materials.

Quantitative Cellulase Activities Screening Assays of Trichoderma Isolates

It is always a challenge to determine the total cellulase activity efficiently without reducing accuracy. The most commonly used test for many microorganisms for total cellulase activity detection is the FPA established by the IUPAC [40]. In this work, it was possible to detect the synergic effect of cellulase complex in all of the fungal isolates of Trichoderma genus. This synergic effect was determined by FPA activity that allowed a rapid evaluation of cellulases

acting on cellulose [41].

All the isolates showed FPA enzymatic activity during the incubation days. The LBM103 isolate showed the highest FPA activity, with significant statistical differences, mainly in the third and fourth days (Figure 2). The most widely accepted mechanism of enzymatic hydrolysis proposes that synergistic cooperation of EGs, CBHs and BGLs is a prerequisite for efficient degradation of cellulose [5,42,43]. Thus, when synergism is lacking, an incomplete hydrolysis can occur due to an incomplete cellulase system or an insufficient enzyme loading [44- 46].

Conclusion

Sazci et al. [31] and Florencio et al. [38] reported the correlation between qualitative screening with Congo red technique and quantitative screening with DNS reagent method [30], but not reported the composition of the medium to use for it. The standardization of a solid medium to detect qualitative cellulase activities with Mandels medium as a nitrogen complex at pH 4, CMC as only carbon source and Congo red technique can be used in a rapid, sensitive and reproducible way for screening cellulase-producing fungi. In this study, the selected solid medium could be comparable with quantitative FPA activity in four isolates of Trichoderma genus. As a consequence, Mandels medium (as a nitrogen complex) amended with CMC as only carbon source at pH 3.5, and the Congo red technique can be used in a rapid, sensitive and reliable way for screening cellulase-producing fungi.

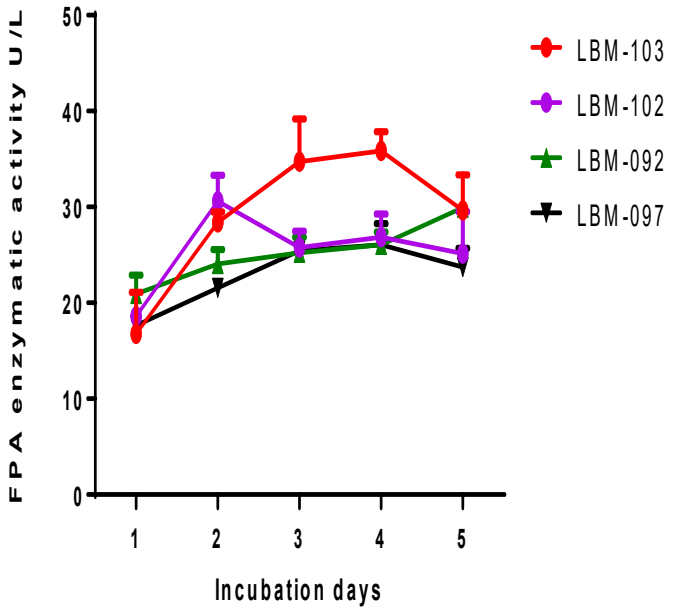

Figure 2: FsPA enzymatic activity of the fungal isolates of Trichoderma genus during five incubation days. The FPA activity was determined by Ghose [29] and the DNS method [30].

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Conflict of Interest

The authors of this manuscript declare there is not any conflict of interest.

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