

Full Length Research Paper

A simple and rapid plate assay for screening of inulin-degrading microorganisms using Lugol's iodine solution

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In this report, a simple and rapid agar plate assay was established for screening of halophilic, inulin-degrading microorganisms. Two strains considered inulinolytic with this method were chosen and the inulinolytic activities in their culture supernatant were measured with the Somogyi-Nelson method, while their hydrolysis products of inulin were detected with TLC chromatogram.

Key words: Screening, halophilic microorganism, inulinase, Lugol's iodine solution.

INTRODUCTION

Plate screening assay is commonly used for detection of extracellular hydrolytic enzymes produced by microorganisms. To obtain inulin-degrading microorganisms from the environment, efficient plate screening methods are prerequisite. So far, three kinds of plate assays for screening inulin-degrading microorganisms had been described in the literatures. The most common useful plate technique is based on the ability of microbial growth on agar plate with pure inulin as the sole carbon source (Allais et al., 1986). As most of the inulin extracted from plants are practical grade and usually contain low concentration of other organic impurities, some isolates may use these substrates as carbon sources instead of inulin, which may lead to false positive result. In the second plate assay, the existence of inulin-hydrolyzing enzyme in agar plates can be detected by a clear zone surrounding the bacterial colony after leaving the plates at 4°C for 1 week (Vullo et al., 1991). Inulin has a low solubility at such low temperature, while the products of inulin hydrolysis are soluble.

This method is time-consuming, and is not suitable for large scale screening programs. In the third method, as

described by Castro et al. (1995) and Ten et al. (2004, 2007), dye-labeled inulin was incorporated into the agar medium and inulinolytic activity was detected by the disappearance of the color around the positive colonies, forming distinct clear zones. In this report, we described a simple, rapid and sensitive agar plate method which can be used for the rapid screening inulin-degrading microbes from environment samples. Inulin compared with the third established method, no high purity inulin or dye-labeled inulin is needed in this study, and the screening time is shortened from 12-24 h to 1-2 h.

MATERIALS AND METHODS

Brine and sediment samples were collected from marine solar salterns in Jiaozhou Bay (36°7'24.44"N, 120°14'44.3"E), China. 0.1 ml of serial diluted samples dissolved in sterilized 4% (w/v) NaCl solution were spread on MHI agar plates which contained inulin (BBI, Canada) as the carbon source and incubated at 37°C for 3 to 5 days to obtain colonial growth. MHI, a modified MH medium (Rodríguez et al., 1980) contained the following components (g L⁻¹): inulin, 20; NaCl, 79.45; KNO₃, 0.1; MgSO₄·7H₂O, 10.45; MgCl₂·6H₂O, 6.9; KCl, 2.1; CaCl₂·6H₂O, 1.5; K₂HPO₄, 0.05; NaHCO₃, 0.2; NaBr, 0.25 and trace element solution, 2 ml. The pH values were adjusted from 7.2 to 7.5, and Bacto agar (18 g L⁻¹) was added if necessary. The trace element solution contained (g L⁻¹): EDTA·2Na 5.2; FeCl₂·4H₂O 1.5; CoCl₂·6H₂O 0.19; MgCl₂·4H₂O 0.1; ZnCl₂ 0.07; H₃BO₃ 0.06; NaMoO₄·2H₂O 0.04; CuCl₂·2H₂O 0.02; and ferric ammonium citrate 3. The medium were sterilized at 110°C for 30 min. The trace element solution and K₂HPO₄ was filtered with 0.22 µm cellulose acetate filter, while inulin was sterilized

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separately and added before pouring the medium into the plates. 0.1 ml of serial diluted sediment or brine samples in sterile 4% NaCl solution were spread on MHI agar plates and the plates were incubated at 37°C for 72 to 96 h. Following incubation, plates were flooded with Lugol's iodine solution (1.5% KI, 1% I₂) for 3 to 5 min.

Subsequently, the staining solution was poured off and the plates were washed two times with suitable amount of distilled water. Then the agar plates were kept open at room temperature for 15 to 60 min. Flooding inulin plates with Lugol's iodine solution results in brown plates with colorless zones where the inulin has been degraded. Strain forming a clear halo around the colony was recorded as inulinolytic, whereas strain that did not form halo was recorded as noninulinolytic. In addition, the ratio of the hydrolytic zone diameter to the colony diameter (H/C) for each inulinolytic strain was calculated at the same time. Total genomic DNA extraction and amplification of 16S rDNA gene from the bacteria isolates were performed according to the methods described by Nathan et al. (2004). Total genomic DNA extraction and amplification of ITS sequence, 18S rDNA and 5.8S rDNA genes from fungi isolates were carried out as described by Plaza et al. (2004). To check the consistency of forming halos on inulin-agar plate with the production of inulinolytic enzymes, strains (LS-A18, MH-F19) showing hydrolytic zones and other 10 bacterial isolates without hydrolytic zones on inulin-agar plates were selected. They were inoculated into a 250 ml shake flask containing 50 ml of MHI medium respectively, and cultured at 37°C and 180 rev min⁻¹ for 80 h on a rotary shaker.

Afterwards, the fermented liquid broth was centrifuged at 5,250×g and 4°C for 10 min, and the supernatant was used as the crude enzyme preparation. The inulinase activity was assayed by measuring the amount of reducing sugar released with inulin from dahlia (Sigma, USA) as the substrate. 0.2 ml of suitably diluted crude enzyme was mixed with 1.8 ml of 2% (w/v) inulin in 0.1 mol L⁻¹ Tris-HCl buffer, pH 9.0. After the mixture was incubated at 55°C for 10 min, the reducing sugar produced was measured by the Nelson method (Nelson, 1944) as modified by Somogyi (1952), and the potential existence of reducing sugar in the crude enzyme solution was subtracted with thermally inactivated crude enzyme as the control. One unit of inulinase activity (U) was defined as the amount of enzyme that produced reducing sugar equivalent to 1 μmol of fructose per min. In addition, further examination of hydrolytic products by the crude enzyme from strains LS-A18 and MH-F19 was qualitatively identified by thin-layer chromatography (TLC). Samples of 3 μl hydrolyzate were spotted on the pre-coated TLC plates (Silica gel 60, Merck, Germany). The plates were developed two times with *n*-butanol-isopropanol-acetic acid-water (7:5:4:2) as the ascending development solvent. The reducing sugars were visualized by spraying with a reagent containing 2 ml aniline, 2 g diphenylamine dissolved in 100 ml methanol, and 15 ml 85% phosphoric acid. Glucose and fructose were used as sugar standards.

RESULTS AND DISCUSSION

A total of 376 microbial strains were isolated on MHI-agar plates with inulin from chicory (BBI, Canada) as the sole carbon source. After the plates were flooded with Lugol's iodine solution, 51 isolates formed clearing zones differing in size around their colonies. Among them, one bacterial strain LS-A18 and one actinomycete strain MH-F19 forming clear hydrolytic zones (Figure 1) were selected and identified as *Marinimicrobium koreense* and *Nocardiopsis lucentensis* based on their morphological, partial biochemical identification and 16S rDNA sequence analysis. The 16S rRNA sequences were deposited at

GenBank database with accession numbers of HQ882175 and HQ882176, respectively. One fungi isolate, strain ZJ-T2, forming hydrolytic zone (Figure 1) was also selected and identified as *Penicillium chrysogenum* (GenBank accession number HQ882177).

The inulinase activities of strains LS-A18 and MH-F19 in their supernatant of fermented broth were measured to be 12.5 and 9.6 U ml⁻¹, respectively; while no reducing sugar was detected for the other 10 isolates without forming halos on inulin-agar plates. As shown in Figure 2, the crude inulinolytic enzyme from strain LS-A18 hydrolyzed inulin into fructose and glucose, while the inulinolytic enzyme from strain MH-F19 hydrolyzed inulin into fructose and FOS with various degrees of polymerization. This result implied that strain LS-A18 only produced exo-inulinase(s), while strain MH-F19 might produce endo-inulinase or exo- and endo-inulinase(s) in their culture supernatant liquid. According to the method introduced by Vullo et al. (1991), inulinase activity can be detected by leaving the inulin agar plates at 4°C for about 2 weeks, so that a clear zone surrounding the colony could be observed. In our study, no halos could be observed even that the concentration of inulin in the medium increased up to 5% (w/v) and the plates were left at 4°C for 2 weeks. This phenomenon was also observed in the process of screening inulin-degrading, marine-derived yeasts from marine environment (Zhen-Ming Chi, private communication). It may be possible that the composition of medium, especially relative high-salinity in the medium inhibited the formation of hydrolytic zone on agar plate. Interestingly, all inulinases of microbial origin reported had optimal activity at the acid or neutral condition (pH 3.0 to 7.0) (Singh and Gill, 2006), whereas we found the optimal pH of the crude enzyme preparation from *M. koreense* LS-A18 was at 9.0. These results imply that cells of *M. koreense* LS-A18 produce a novel, extracellular alkaliphilic inulinase in MHI medium, which has never been reported before this study.

Therefore, purification and characterization of the inulinase from strain LS-A18 are being undertaken in our laboratory. Since pure inulin (Sigma, USA) is very expensive, and is not suitable for large scale screening programs, two other kinds of practical grade inulin (90% purity), inulin from chicory (BBI, Canada) and inulin from Jerusalem artichoke (Wenyuan Co. Ltd., Yucheng, China) were chosen as the carbon source, and the screening results coincided very well (100%) with those using inulin from Dahlia (98% purity) as carbon source. From a practical point of view, practical grade inulin can be used as the carbon source instead of inulin with high purity. In addition, keeping the plates open after staining is a very important step to further enhance the visibility of the inulinolytic zones, and usually increase the contrast between degradation zones and background within 1 to 2 h (Figure 3). The staining solution in this method contains iodine and is toxic to the colonies on the plate. It can be overcome by prior replicating plates.

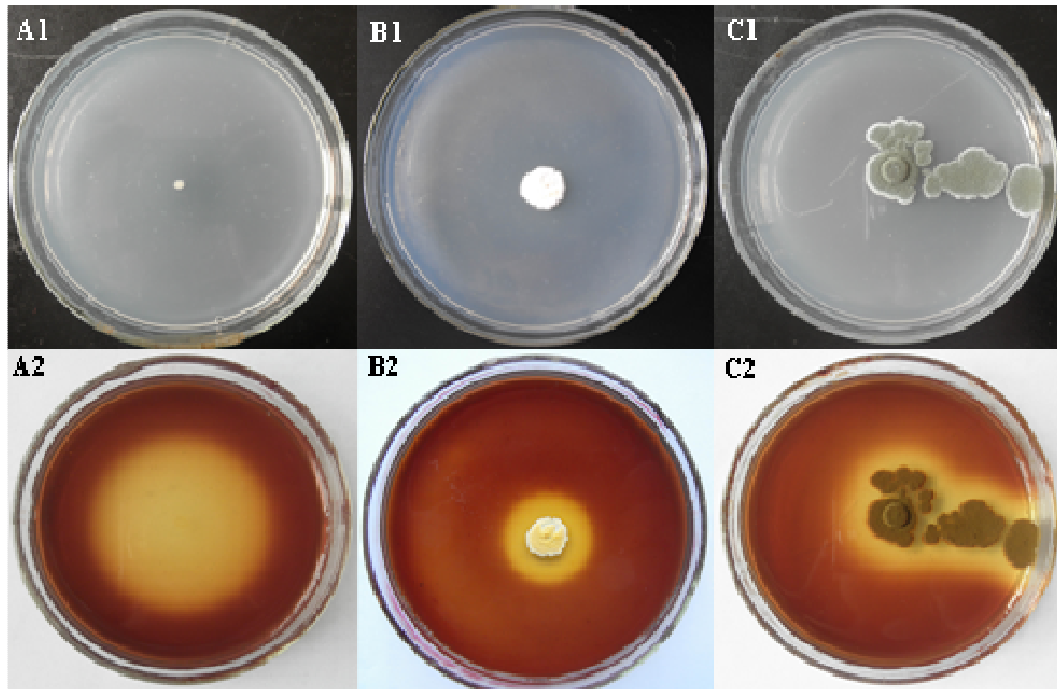


Figure 1. Screening of inulin-degrading microorganisms on MHI agar plates stained with Lugo's iodine solution. A) Colony of *M. koreense* LS-A18 before (A1) and after (A2) stained; B) Colony of *N. lucentensis* MH-F19 before (B1) and after (B2) stained; C) Colony of *P. chrysogenum* ZJ-T2 before (C1) and after (C2) stained.



Figure 2. TLC chromatogram of hydrolysis products of inulin by inulinolytic enzymes from *M. koreense* LS-A18 and *N. lucentensis* MH-F19. Lane 1 glucose, Lane 2 fructose, Lane 3 inulin (Sigma); Lane 4 hydrolysis products by inulinase from strain LS-A18; Lane 5 hydrolysis products by inulinase from strain MH-F19. Volume of samples 3 μ l.

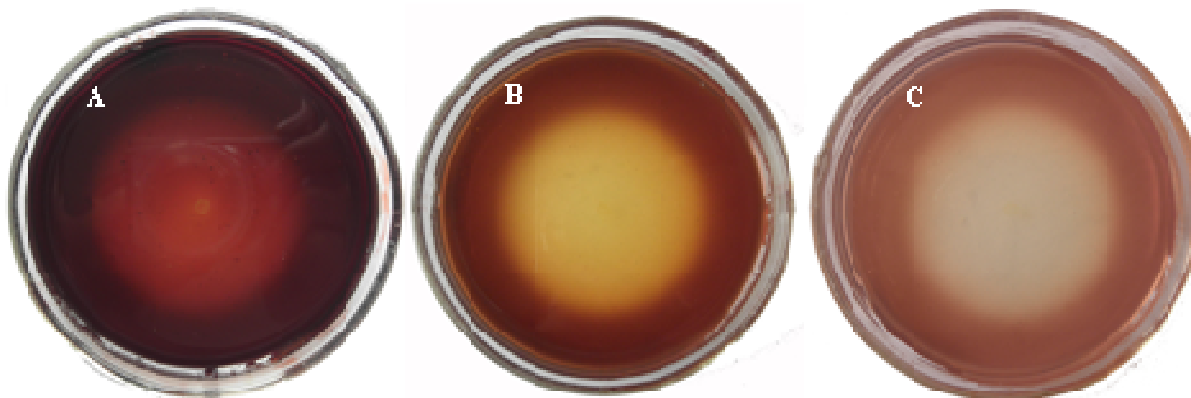


Figure 3. The hydrolytic zones of *M. koreense* LS-A18 formed on MHI agar plate after stained with Lugol's iodine solution for 15 min (A), 60 min (B), and 180 min (C).

Subsequently, colonies forming hydrolytic zones can be retrieved from the master plates. It allows for screening a large number of inulin-hydrolyzing colonies from environment samples in a short time, while no dye-labeled substrates are needed at the same time. In addition, this method has proven very useful in the isolation of microorganisms from marine or other high-salinity environments. Furthermore, it would also have application in rapid screening mutant strains after treating with physical, biochemical or biological mutagenesis. To our best knowledge, this is the first report on the use of Lugol's iodine for the detection of inulin-degrading microorganisms.

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REFERENCES

- Allais JJ, Kammoun S, Blanc P, Girard C, Baratti JC (1986). Isolation and characterization of bacterial strains with inulinase activity. *Appl. Environ. Microbiol.* 52: 1086-1090.
- Castro GR, Baigori MD, Sineriz F (1995). A plate technique for screening of inulin degrading microorganisms. *J. Microbiol. Meth.*, 22: 51-56.
- Nathan AM, Jessica MK, Valerie B, Martin D, David HS (2004). Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. *Appl. Environ. Microbiol.*, 70: 7520-7529.
- Nelson N (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.*, 153: 375-380.
- Plaza GA, Upchurch R, Brigmon RL, Whitman WB, Ulfing K (2004). Rapid DNA extraction for screening soil filamentous fungi using PCR amplification. *Polish J. Environ. Stud.*, 13: 315-318.
- Rodriguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A (1980). Isolation of extremely halophilic bacteria able to grow in defined inorganic media with single carbon sources. *J. Gen. Microbiol.*, 119: 535-538.
- Singh P, Gill PK (2006). Production of inulinases: recent advances. *Food Technol. Biotech.*, 44: 151-162.
- Somogyi M (1952). Notes on sugar determination. *J. Biol. Chem.*, 195: 19-23.
- Ten LN, Im WT, Kim MK, Kang MS, Lee ST (2004). Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *J. Microbiol. Meth.*, 56: 375-382.
- Ten LN, Im WT, Aslam Z, Larina L, Lee ST (2007). Novel insoluble dye-labeled substrates for screening inulin-degrading microorganisms. *J. Microbiol. Meth.* 69: 353-357.
- Vullo D, Coto CE, Sineriz F (1991). Characteristics of an inulinase produced by *Bacillus subtilis* 430A, a strain isolated from the rhizosphere of *Vernonia herbacea* (Vell Rusby). *Appl. Environ. Microbiol.*, 57: 2392-2394.