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A rapid, sensitive, simple plate assay for detection of microbial alginate lyase activity

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ABSTRACT

Screening of microorganisms capable of producing alginate lyase enzyme is commonly carried out by investigating their abilities to grow on alginate-containing solid media plates and occurrence of a clearance zone after flooding the plates with agents such as 10% (w/v) cetyl pyridinium chloride (CPC), which can form complexes with alginate. Although the CPC method is good, advantageous, and routinely used, the agar in the media interferes with the action of CPC, which makes judgment about clearance zones very difficult. In addition, this method takes a minimum of 30 min to obtain the zone of hydrolysis after flooding and the hydrolyzed area is not sharply discernible. An improved plate assay is reported herein for the detection of extracellular alginate lyase production by microorganisms. In this method, alginate-containing agar plates are flooded with Gram's iodine instead of CPC. Gram's iodine forms a bluish black complex with alginate but not with hydrolyzed alginate, giving sharp, distinct zones around the alginate lyase producing microbial colonies within 2–3 min. Gram's iodine method was found to be more effective than the CPC method in terms of visualization and measurement of zone size. The alginate-lyase-activity area indicated using the Gram's iodine method was found to be larger than that indicated by the CPC method. Both methods (CPC and Gram's iodine) showed the largest alginate lyase activity area for *Saccharophagus degradans* (ATCC 43961) followed by *Microbulbifer mangrovi* (KCTC 23483), *Bacillus cereus* (KF801505) and *Paracoccus* sp. LL1 (KP288668) grown on minimal sea salt medium. The rate of growth and metabolite production in alginate-containing minimal sea salt liquid medium, followed trends similar to that of the zone activity areas for the four bacteria under study. These results suggested that the assay developed in this study of Gram's iodine could be useful to predict the potential of microorganisms to produce alginate lyase. The method also worked well for screening and identification of alginate lyase producers and non-producers from environmental samples on common laboratory media. They did this by clearly showing the presence or absence of clearance zones around the microbial colonies grown. This new method is rapid, efficient, and could easily be performed for screening a large number of microbial cultures. This is the first report on the use of Gram's iodine for the detection of alginate lyase production by microorganisms using plate assay.

1. Introduction

Alginate is a linear polysaccharide in which β-D-mannuronate (M) and α-L-guluronate (G) are covalently (1–4)-linked in different sequences [1]. Alginates are quite abundant in nature. In brown algae, they are produced as a structural component, comprising of up to 40% of dry weight [2]. Commercial alginates are produced by extraction from marine macroalgae such as *Laminaria hyperborea*, *Macrocystis pyrifera*, *Laminaria digitata*, *Ascophyllum nodosum*, *Laminaria japonica*, *Eclonia maxima*, *Lessonia nigrescens*, *Durvillea antarctica*, and *Sargassum* sp. Some bacteria can also synthesize alginates [3,4].

Alginate lyases are a group of enzymes that can degrade alginate through β-elimination of the glycosidic bond [5–7]. They yield various oligosaccharides with unsaturated uronic acid at the non-reducing terminus, and unsaturated uronic acid monomers. Various alginate lyases have been found and isolated from algae, marine invertebrates, and marine (and some soil) microorganisms. Alginate lyases are useful as pharmaceuticals for enhancing antibiotic effect on mucoid *Pseudomonas aeruginosa* in cystic fibrosis [8]. They have been widely used in many applications, including production of bioactive oligosaccharides, control of polysaccharide rheological properties, and polysaccharide structure analysis [9].

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Alginate oligosaccharides produced using alginate lyases exhibit some interesting biological activities useful in therapeutics [10] and biotechnology [11]. These include such as prebiotic, stimulation of the secretion of cytotoxic cytokines from human macrophage [12,13], induction of the granulocyte colony-stimulating factor [14], and plant growth promotion [15,16]. Endolytic and exolytic alginate lyases have the ability to convert alginate into unsaturated monosaccharide by the saccharification process; therefore, alginate lyases have potential as key biocatalysts for application in renewable sources of biochemicals and biofuels in the near future [17].

Different microorganisms have been reported to produce alginate lyase and degrade alginate (e.g., *Pseudomonas* sp. OS-ALG9 [18], *Pseudoalteromonas elyakovii* IAM [19], *Sphingomonas* sp. A1 [20], *Agrobacterium tumefaciens* [21], *Saccharophagus degradans* [22], and *Microbulbifer mangrovi* [23]). Alginate lyases produced by microorganisms have potential applications in industry, medicine, agriculture, and biotechnology. Therefore, different groups of microorganisms need to be screened to understand their potential for production of alginate lyase. In this regard, qualitative screening methods are useful because they can give a sense of the potential of microorganisms to produce the enzyme. A few qualitative methods have been reported for such studies using plate assays. Researchers in one such study described the use of 0.1% polyguluronate and polymannuronate from alginate, as substrate in a solidified agarose medium for bacterial growth on plates. The clear zone was produced by enzymatic digestion of substrate in the medium, and the unhydrolyzed substrate was stained with either cetyl pyridinium chloride (CPC) or ruthenium red [24]. Takeshita et al. [25] developed an improved method based on tandem use of a ubiquitous reagent, calcium chloride, together with 70% ethanol as a precipitant for undigested alginate, and polyguluronate in the medium. Yoshida et al. [26] used covalently dye-labeled substrates for measurement of polysaccharide-degrading enzyme activities. Baron et al. [27] used 1.5% agarose and 1% G-rich sodium alginate from *L. hyperborea* in a plate assay to detect the *Klebsiella pneumoniae* G-specific lyase overexpressed in *Escherichia coli*. Colonies were flooded with 95% ethanol, and clear zones around the lyase-producing clones were observed against a background of opaque, milky white, precipitated alginate. Other variations included the use of dilute HCl [28] or calcium chloride to precipitate the undigested alginate [29,30] or the incorporation of charcoal into the media in tubes [31] or plates [32] for better visualization of lyase activity. Schiller et al. [33] described another plate assay in which selected *P. aeruginosa* or *E. coli* clones that expressed lyase activity, displayed depressions of $\geq$1 cm in the medium surrounding the colonies. Kitamikado et al. [34] described a turbidimetric assay based on the fact that acidic mucopolysaccharides combines with bovine serum albumin in acidic solutions to develop white turbidity, and that alginate degradation by lyase prevents this turbidity from occurring. The presence of alginate lyases has also been identified using isoelectric focusing and a substrate-overlay technique [35–37], which detected multiple forms of lyase in crude bacterial extracts.

Most of the qualitative assays described above incorporate alginate within solid growth media and can be used to detect alginate lyase production by bacterial colonies. The ability of bacteria to effectively release or secrete the enzyme can influence the detectability of lyase activity in these assays. Of these methods, plate assays are simple and user friendly. The plate assay of CPC is most routinely used for detection of alginate lyase production by microorganisms [9,23], and it is particularly advantageous for colorful media. However, agar in the media interferes with the action of CPC, making visual determination of the clearance zones very difficult [24]. Some researchers suggested using highly purified, clarified agarose in place of agar, to enable better clearance zone visualization [1,24]. In addition, this method requires a relatively long time to observe results.

Isolation and screening of microbes with potential to produce alginate lyase is of immense importance keeping in view the demand for new enzymes and the improvement of their biotechnological applications. To facilitate the screening of a large number of alginate lyase-producing microorganisms, it is important to develop a simple assay to screen more samples in shorter time, with chemicals commonly available in laboratories. With these points in mind, we developed a simple, rapid, inexpensive, time saving, and environmentally friendly method for detection of alginate lyase activity. This resulted from investigation of four different bacteria, *S. degradans* (ATCC 43961), *M. mangrovi* (KCTC 23483), *Bacillus cereus* (KF801505), and *Paracoccus* sp. LL1 (KP288668), grown on minimal sea salt medium. The results of the assay were validated by investigating the growth rates of the microorganisms and production of reducing sugars by the DNS method and thin layer chromatography of the liquid broths. The applicability of the plate assay was also tested for environmental samples.

2. Materials and methods

2.1. Chemicals

CPC (2674) was purchased from Daejung Chemicals (Korea). Sodium alginate (A3148) was from Samchun Chemicals (Korea), and Gram's iodine solution (HT902) was from Sigma (St. Louis, USA).

2.2. Microorganisms and maintenance of microbial cultures

The microbial strains, *S. degradans* (ATCC 43961) and *M. mangrovi* (KCTC 23483) were obtained from the American type culture collection and Korean collection for type cultures, respectively. Those in our lab isolated *B. cereus* (KF801505) [38] and *Paracoccus* sp. LL1 (KP288668). All these microbial cultures were maintained on minimal sea salt media [38] consisting of 23 g/L of instant ocean sea salts (Aquarium Systems, Mentor, OH), 1 g/L of yeast extract, 50 mM Tris buffer (pH 7.4), 0.5 g/L of NH_4Cl, Glucose 20 g/L and 1.8% agar. The liquid cultures were prepared by inoculation of single colonies of each of the four bacteria (*S. degradans*, *M. mangrovi*, *B. cereus*, and *Paracoccus* sp. LL1) in minimal sea salt liquid medium [38] in separate flasks, and grown overnight at 30 °C in a shaking incubator (Jeio Tech SI600R, Korea).

2.3. Plate assay for alginate lyase activity

Five microliters of cultures grown overnight of the four bacteria in minimal sea salt liquid medium were spot inoculated at the center of minimal sea salt agar plates fortified with 0.2% sodium alginate, in triplicate, for two separate sets of experiments. The prepared plates were incubated at 30 °C for 48 h. After 48 h, the first sets of plates were flooded with 10% (w/v) CPC and kept for 30 min as suggested by Gacesa and Wusteman [24]. The second sets of plates were flooded with Gram's iodine for 2–3 min.

2.4. Determination of cell growth

The cell growth of the four bacterial species was investigated by culturing them in minimal sea salt liquid medium containing 5 g/L alginate. The insolubility of alginate substrates in the medium interfered with measurement of the growth by spectrophotometry; therefore, cell growth was determined based on the concentration of bacterial cellular protein as per Desvaux et al. [39]. Total solution protein content was taken as a measure of bacterial protein content. To accomplish this, specifically, 0.2 mL of culture broth was centrifuged at 16,000 rev/min for 5 min at 4 °C. The obtained cell pellet was washed with 2.3% (w/v) NaCl solution. Then, 0.2 mL of 0.2 N NaOH was added to the washed cell pellet and the sample was placed in a thermo-mixer at 95 °C and 850 rev/min for 10 min. The sample was cooled in an ice bath for 3 min, centrifuged again and 100 μL of supernatant was collected. The protein concentration was measured using a commercial bicinchoninic acid assay (Sigma–Aldrich) at 562 nm using crystalline bovine serum albumin (BSA) as a protein standard. The experiments were carried out in triplicate and the analysis results presented as the average and standard deviations from the values obtained.

2.5. Reducing sugar assay

The four bacterial species were grown in minimal sea salt liquid medium containing 5 g/L of alginate. The reducing sugars produced by the bacteria were estimated using the 3,5-dinitrosalicylic acid (DNS) method [40]. The supernatants of the culture medium were mixed with DNS solution and boiled for 5 min. Absorbance of