

Simplified Method of Preparing Colloidal Chitin Used For Screening of Chitinase- Producing Microorganisms

N Murthy, B Bleakley

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Abstract

A simplified and efficient method of preparing colloidal chitin from inexpensive crab shell flakes was developed. It modifies some steps of existing techniques to provide significant saving in effort and materials for colloidal chitin preparation. In colloidal chitin preparation, unless the initial chitin flakes are ground to a fine powder, it becomes difficult to later separate the chitin chunks from the precipitated colloidal chitin. The modified technique reported here involves the use of simple everyday lab materials to extract colloidal chitin from crab shell flakes, without the need of powdering the chitin flakes to a uniformly fine size. Utility of the colloidal chitin obtained was shown by using it in plate assays to screen for extracellular chitinase producers, and labeling it with Remazol Brilliant Blue (RBB) for chitinase assays.

INTRODUCTION

Chitin is widespread in both terrestrial and aquatic environments, as a component of invertebrate exoskeletons, fish scales, and cell walls of many fungi. Only cellulose is more globally abundant as a biological polymer (reviewed by Shahidi et al., 1999; and Zaku et al., 2011). Chitin is the most abundant biopolymer in marine environments (Souza et al., 2011). A variety of microorganisms produce extracellular chitinases that can break down chitin (reviewed by Howard et al., 2003; Gohel et al., 2006; and Dahiya et al., 2006). To isolate such microorganisms, chitin is often used in solidified agar media, where clearing zones surrounding microbial colonies indicate production of extracellular chitinase. Because chitin is not readily water soluble, chitin is often chemically modified to form colloidal chitin, with a small particle size that is more readily manipulated to obtain homogenous distribution in agar media, compared to use of physically modified, finely ground chitin that can be difficult to obtain. Lingappa and Lockwood (1961; 1962) devised a colloidal chitin preparation method that was later modified by Hsu and Lockwood (1975). These are referred to in this paper as Lockwood protocols.

The Lockwood protocols have been widely used for isolation of chitinase-producing culturable microorganisms. Modifications of the Lockwood protocols have been developed and used by various researchers since (Gomez Ramirez et al., 2004; O'Risal, 2008), depending on their

research needs. No matter the specific goals, modification of protocols to save time or materials is of continuing interest to researchers. In our studies to characterize a variety of culturable microorganisms for chitinase activity, we arrived at a modified version of the Hsu and Lockwood protocol (1975) combining it with some steps from the protocol of Gomez Ramirez et al. (2004) to save effort and materials. We report in this note on a modified method for colloidal chitin preparation from crab shell flakes that save effort and materials compared to some previous methods.

MATERIALS AND METHODS

A modified protocol of Hsu and Lockwood (1975) was used for the preparation of colloidal chitin. Crab shell flakes (Neptune's Harvest, MA, USA) were manually ground in a mortar and pestle for 5 minutes, then sieved through the top piece of 130 mm two piece polypropylene Buchner filter. Twenty grams of the sieved crab shell flakes were then treated with 150 ml of ~12M concentrated HCl (BDH Aristar) in a 1000 ml beaker. The HCl was added slowly with continuous stirring with the use of a glass pipette for 5 minutes, followed by stirring for 1 minute at an interval of every 5 minutes for 60 minutes in a chemical fume hood at room temperature (25 ° C). The chitin- HCl mixture was then passed through 8 layers of cheesecloth to remove large chitin chunks.

The clear filtrate obtained (100 ml) was then treated with 2 liters of ice cold distilled water to allow precipitation of

colloidal chitin. This was incubated overnight under static conditions at 4°C to facilitate better precipitation of colloidal chitin. This was later passed through two layers of coffee filter paper, housed in a Buchner funnel (130 mm) seated in a vacuum filtration flask under vacuum. Approximately 3 liters of tap water (pH of ~8.0) were passed through the colloidal chitin cake using this filter assembly, until the pH of the filtrate had risen to 7.0 (estimated by pH paper). The colloidal chitin obtained was pressed between coffee filter papers (to remove additional moisture), and then placed in a 100 ml glass beaker covered with two layers of aluminum foil and sterilized by autoclaving at standard temperature and pressure (STP) (15 psi, 20 minutes, 121 ° C).

The autoclaved colloidal chitin was stored at 4°C until further use in moist form, based on the protocol of Gomez Ramirez et al. (2004) that used moist colloidal chitin. The colloidal chitin we obtained had a soft cake-like texture.

Modified protocols combining elements from Gomez Ramirez et al. (2004) and Hsu and Lockwood (1975) were used for the preparation of chitin agar, colloidal chitin broth (CCB), and RBB-stained colloidal chitin broth (RBB-CCB). In the preparation of chitin agar, instead of 0.2% dry powdered colloidal chitin, 2.0% moist colloidal chitin was used. The colloidal chitin agar (pH 7.0+/- 0.2) prepared had the following ingredients (in g/L): (K_2HPO_4 , 0.7; KH_2PO_4 , 0.3; $MgSO_4 \times 5H_2O$, 0.5; $FeSO_4 \times 7H_2O$, 0.01; $ZnSO_4$, 0.001; $MnCl_2$, 0.001), amended with 2.0 % moist colloidal chitin. Bacto Agar (Difco) was added at 2.0% as a solidifying agent. The medium was sterilized by autoclaving at STP and poured into sterile Petri plates (approximately 20 ml per plate). The protocol for RBB-stained colloidal chitin (RBB-CC) preparation was followed as per Gomez Ramirez et al. (2004) with the use of alum and $FeSO_4 \times 7H_2O$ as mordants. The RBB-CCB was prepared as per Gomez Ramirez et al. (2004), with 2.0 % RBB-CC as chitin source; otherwise all other recipe components and steps were as described above, except agar was omitted. For preparation of colloidal chitin broth (CCB), 2% moist colloidal chitin (non-stained) was used as chitin source. The CCB otherwise had the same pH and recipe as for RBB-CCB.

To act as a positive control for plate assay of chitinase activity, 10.0 microlitres of a 24-hour culture of *Serratia marcescens* (strain 361; Presque Isle Cultures; Erie, PA; USA) in Tryptic Soy Broth (TSB; Difco) was inoculated onto chitin agar at 0.6 OD at 600 nm using spot inoculation. This was incubated at 30°C for 7 days. Two plates were

used, with each plate having three spots. To act as a positive control for broth assay of chitinase activity, 100.0 microlitres of a 24 hour culture of the *S. marcescens* in Tryptic Soy Broth (TSB) was inoculated into RBB-CCB and CCB (4 ml in 25 ml scintillation vials) at 0.6 OD at 600 nm. This was incubated for 2 days at 25 +/- 0.5°C on a rotary shaker at 100 rpm. For RBB-CCB and CCB, four replicates were used. After incubation, the *S. marcescens*, cultures were observed for evidence of chitinase production. On chitin agar, the diameter of the colony and the diameter of the clearing zone was measured in millimeters using a metric ruler. For the RBB-CCB assay, 2.0 ml of the culture was centrifuged at 8000 rpm at room temperature for 5 minutes. Then 1.0 ml of the supernatant was carefully transferred into disposable plastic cuvettes, and the absorbance was read at 595 nm (Thermo Spectronic: Genesys 20, model 4001/4).

Assay of *S. marcescens* grown in CCB was conducted similarly. To minimize the effect of pigment production on absorbance values, the absorbance values obtained from the culture filtrate of CCB was subtracted from the absorbance values obtained from the culture filtrate of RBB-CCB.

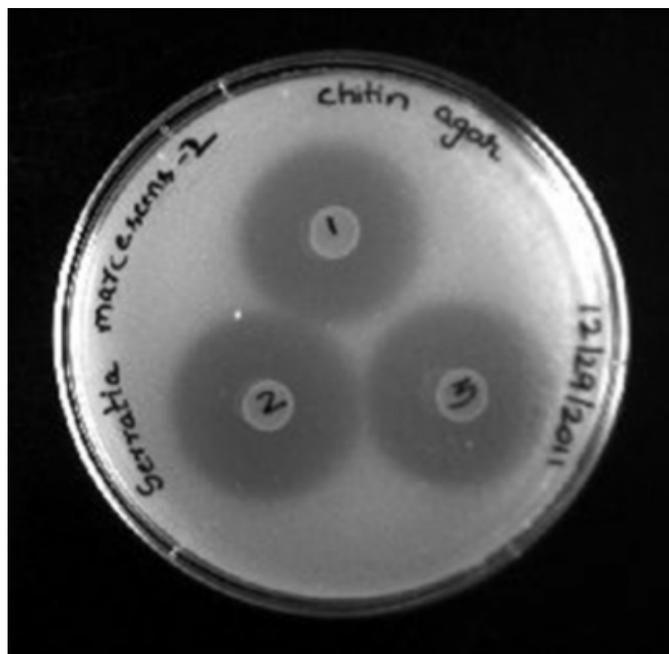
RESULTS

Plate assay: On colloidal chitin agar, *S. marcescens* produced large chitin clearing zones around the colonies in all the replicates (Fig 1); indicating the production of chitinase. The clearing zone was 24.5 +/- 1.048 standard deviation (mm) in diameter. The ratio of the clearing zone to colony diameter was 3.094.

Broth Assay: In the RBB-stained colloidal chitin broth, chitinase production by *S. marcescens* was indicated through the release of RBB dye into the broth medium. Absorbance was measured at 595 nm using the sterile control broth as blank. The positive control produced an absorbance of 0.233+/- 0.012 standard error. Based on ANOVA ($p < 0.05$), significant production of chitinase enzyme in broth was found for the positive control using *S. marcescens*.

Figure 1

Fig 1: colonies that produced clearing zones on colloidal chitin agar.



DISCUSSION

Chitin and chitinases are of continuing interest to a variety of researchers (Bhattacharya et al., 2007). Some recipes that describe preparation of colloidal chitin agar omit describing how to obtain and store the colloidal chitin. We wanted to make the preparation procedure as simple and conservative of money and materials as we could manage. The modified protocol we obtained recommends some modifications to the Hsu and Lockwood (1974) protocol for preparing colloidal chitin.

In colloidal chitin preparation, unless the initial chitin flakes are ground to a fine powder, it becomes difficult to later separate the chitin chunks from the precipitated colloidal chitin. The modified technique reported here involves the use of simple everyday lab materials to prepare colloidal chitin from crab shell flakes, without the need of completely grinding the chitin flakes to a uniformly fine size.

The filtration of chitin chunks from the chitin-HCl mixture through cheesecloth saved significant effort; this was one major change to the previous protocols. We are not aware of any other published methods of colloidal chitin preparation that use such a cheesecloth filtration step at the point in the preparation that we recommend. Some chitin is lost at this filtration step, but the final colloidal chitin yield is still good.

The use of coffee filters was also important in allowing rapid filtration of water through the chitin cake. Although we were initially unaware of other people using coffee filters in colloidal chitin preparation, we later found that O'Risal has made similar use (2008).

Additionally, the techniques described here provided the advantage of obtaining good quality colloidal chitin at a very reasonable price, using crab shell flakes (30 dollars American for 12 pounds of crab shell flakes, sans shipping) as starting material.

The use of oven-dried colloidal chitin in agar medium has certain advantages in comparison to the moist colloidal chitin, like storage at room temperature. However, after drying, colloidal chitin has to be powdered very finely to obtain a more uniform distribution of colloidal chitin in chitin agar. Hence, skipping the drying step and using the moist colloidal chitin at 2% (weight/volume) can help simplify the procedure. If a lyophilizer is available, then freeze drying of the colloidal chitin might serve to keep the colloidal chitin stable and useful as well or better than the steps described here.

Activity of chitinase on the moist colloidal chitin was demonstrated with the use of a positive control organism, *S. marcescens*. Both in plate and broth assays, production and activity of secreted chitinase enzyme was detected, showing the utility of using the moist colloidal chitin for preparation of chitin agar and RBB-labeled chitin.

We have made use of the colloidal chitin procedures described above to screen a variety of *Bacillus* strains that are used for biological control of *Fusarium* Head Blight for chitinase (data not shown). We have also screened several biofilm isolates from the Homestake gold mine in Lead, South Dakota for chitinase (data not shown). We plan to make regular use of the colloidal chitin to screen our research and teaching culture collections for chitinase producers.

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Author Information

N. K. S. Murthy

Biology/Microbiology Department, South Dakota State University

B. H. Bleakley

Biology/Microbiology Department, and Plant Science Department, South Dakota State University