Supporting Information document

Saccharomyces Cerevisiae as an untapped source of fungal chitosan for antimicrobial action [1]

)

Md. Masirul Afroz[a,b], Md Nayeem Hasan Kashem [a,c], KM Prottoy Shariar Piash[a,d] and Nafisa Islam[a,*]

a Department of Chemical Engineering, Bangladesh University of Engineering and Technology, Dhaka, Bangladesh
b Department of Chemical Engineering, University of Wyoming, Laramie, Wyoming, USA
c Department of Chemical Engineering, Texas Tech University, Lubbock, Texas, USA
d Department of Chemical and Biomedical Engineering, West Virginia University, Morgantown, West Virginia, USA

* Corresponding author: nafisaislam@che.buet.ac.bd

S1. Preparation of culture media

Preparation of Agar Plates

Agar plates for *Aspergillus spp.* were prepared using commercial Potato Dextrose Agar (PDA) medium. Maintaining the ratio recommended by the manufacturer, nutrient agar solution was autoclaved for 20 min. The solution was poured into gas sterilized plastic petri plates inside a biosafety hood (level 2, LCB-0123B-A2, Daihan LabTech Co Ltd, Korea) and left to cool down and solidify for 4-5 min.

Agar suspensions were also prepared using commercial Mannitol Salt Agar Base (for *Staphylococcus aureus* culture) and Mueller Hinton Agar (for antimicrobial efficacy testing) following the same procedure. Mueller Hinton Agar is not specific to any particular bacteria. For agar plate preparation, an agar suspension was made to the ratio specified by the manufacturer. Mannitol Salt Agar Base powder and Mueller Hinton Agar powders were mixed with distilled water and dry sterilized in an electric oven. The respective suspensions were then allowed to cool to about 60°C; 20 ml was poured into Petri dishes, whilst the suspensions remained runny. The Mannitol Salt Agar Base petri dishes (MSA agar plate) and Mueller Hinton Agar petri dishes

[1] Part of the results of this manuscript have been presented in the Fifth International Conference for Chemical Engineering, ICChE 2017.

(MHA agar plate) were left inside the biosafety cabinet for the time taken for the agars to solidify. The plates were kept upside down until further experiments.

Preparation of the Potato Dextrose Broth (PDB) Media

Fresh potatoes were washed and cut into cubes without peeling. The cubes of potatoes were boiled with distilled water for 30 min. The ratio was 0.6 g potatoes/ mL distilled water. The potato infused liquid was collected by filtration and glucose and $NaNO_3$ were added to the filtrate at the ratio 0.1g glucose/mL solution and 0.4 mg $NaNO_3$/mL solution. $NaNO_3$ was used as the nitrogen source for *Aspergillus niger*. The pH was made up to 5.2 with aid of a pH meter. (Hanna Instruments- HI 2211, USA). The prepared Potato Dextrose Broth (PDB) solution was heated to 120^{o}C in a high temperature oven (Barnstead international, Model - 3510-1, USA) as a method of dry sterilization. Typically, the PDB culture media was used within an hour of preparation to prevent any risk of contamination. If storage was required, the media was stored at 4 °C until further use.

S2. Extraction of chitosan from shrimp shells

Washing and de-fleshing. The frozen shrimp shells were rinsed with cold water to wash off any surface dirt. The shells were then immersed in warm distilled water at ~60^{o}C to remove as much lipids and fats as possible. They were then de-fleshed by hand and rinsed under cold water again. The cleaned shrimp shells were then dried in a low temperature oven at 60^{o}C overnight. The dried shells were ground with the use of a mortar and pestle to reduce it to smaller bits, and then a food processor was used to obtain finely powdered shrimp shells.

Deproteination. The washed and dried powdered prawn shells were treated with 5% sodium hydroxide (NaOH) solution (w/v 1:8) at 60^{o}C for 2h to remove the remaining proteins and other organic materials. After the reaction, the solution was colored and frothy, therefore, the sample was washed repeatedly with water until most of the color and frothing disappeared and the resulting solution was near neutral. The sample was finally washed with distilled water and then dried in an oven at 60^{o}C to constant weight.

Decoloration. The powdered shells after treatment with alkali were treated with acetone at room temperature. Enough acetone was added to submerge the shrimp shells, and the setup was kept on an orbital shaker at 150 rpm for 24 hours. The shells were filtered after 24 hours, rinsed with distilled water and dried at 60^{o}C in an oven overnight.

Demineralisation. The deproteinated and decolorized material was treated with a 1% HCl solution (w/v 1:10) for 24 h at 25^{o}C to dissolve the calcium carbonate. The prawn shells were then washed several times with water to remove the calcium carbonate and other water-soluble impurities. The resulting chitin was obtained in the form of a very light brown powder. The content of chitin in prawn shells was determined from the weight differences between the dry weight of the raw materials and the resulting weight of chitin obtained.

Deacetylation of Chitin. Chemical deacetylation was achieved by treatment of extracted chitin with 50% NaOH solution at boiling temperatures of 100^{o}C using a solid to solvent ratio of 1:10. The reaction mixture was kept in the oven for 5 hours. After the reaction, the material produced was washed several times with distilled water until near to neutral pH and dried at 60^{o}C in a vacuum oven until constant weight. The powder thus obtained was sent for characterization.

Yield of chitosan from shrimp shells. Chitosan yields are defined as the mass of chitosan obtained in mg for 1 g of starting raw material. The mass of dry shrimp shells was measured after grinding to powder and the mass of dried chitosan obtained after all the steps involved was also measured. Yield (mg/g shrimp shell) is given by the formula in Equation S1

$$\text{Yield} = \frac{\text{dry mass of chitosan (g)}}{\text{mass of dry shimp shell powder (g)}} \times 1000 \qquad \text{(S1)}$$

S3. Characterization of the Extracted Chitosan using Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy analysis of the chitosan samples was done using a Shimadzu FTIR spectrophotometer. Potassium bromide (KBr) disk method was utilized to obtain the spectra.

The process involved mixing KBr with chitosan samples at nearly 100:1 ratio using a mortar. The mixture was then pressed into a disk and mounted in the spectrophotometer. Frequency range of the spectra was from 4000 to 400 cm^{-1} with 45 scans. The resolution was 2 cm^{-1}. Using the data obtained from FTIR spectroscopy of chitosan samples obtained from shrimp shells, *Aspergillus niger* and *Saccharomyces cerevisiae*, degree of deacetylation was calculated using Equation S2[2]

$$\text{DD \%} = 100 - \left(\frac{A_{1655}}{A_{3450}}\right) \times \left(\frac{100}{1.33}\right) \qquad \text{(S2)}$$

Here, A_{1655} is the value of absorbance at peak near 1655 cm-1,

A_{3450} is the value of absorbance at peak near 3450 cm-1.

FTIR spectroscopy showed results in Transmittance (%). Absorbance was calculated from the Transmittance values using Equation S3.

$$A = 2 - \log (T) \qquad \text{(S3)}$$

S4. Degree of Deacetylation using Acid Base Titration Method

Chitosan samples that were obtained from culture of *A. niger* and *S. cerevisiae* were further characterized using acid base titration method. Values of degree of deacetylation were calculated using Equation S4.

$$DD\ \% = 2.03 \times (V_2 - V_1) / [m + 0.0042 \times (V_2 - V_1)] \qquad \text{(S4)}$$

Here, m is the amount of chitosan used in the titration (0.2 in these experiments),

V_1 and V_2 are the deflection points in the pH curve,

Here,

m, in grams, is the amount of chitosan used in the titration (0.2 g for these experiments),

V_1 and V_2 are the deflection points in the dpH/dV curve.

Coefficient 2.03 results from molecular weight of chitin monomer unit,

Coefficient 0.0042 results from difference between molecular weights of chitin and chitosan monomer units [1]

Coefficient 2.03 results from molecular weight of chitin monomer unit,

Coefficient 0.0042 results from difference between molecular weights of chitin and chitosan monomer units. Dried chitosan extracted from either *A. niger, S. cerevisiae* and shrimp shells was dissolved in 0.1 M HCl at 10% w/v. The solution was titrated using 0.1 M NaOH. The pH of the system was measured and plotted against volume of NaOH, V, during titration. The dpH/dV values were also calculated and plotted for increasing volumes of NaOH.

Using the value of V_1 and V_2 obtained from plotting dpH/dV curve, the degrees of deacetylation of chitosan extracted from *Aspergillus niger*, *Saccharomyces cerevisiae*, and shrimp were calculated using Equation S4.

S5. Biomass growth and identification of *Aspergillus spp.*

The growth characteristics of a sample of *Aspergillus spp.* isolated from moldy onions was investigated using anaerobic surface culture method in Potato Dextrose Broth (PDB) medium. As shown in Fig S1(a), at Day 1 after inoculation, the fungus created a thin and whitish layer on the surface of the medium. Black spores became visible at Day 2 while making the fungal mat comparatively more prominent. Within Day 3 and Day 4, black spores completely covered the top of the fungal mat. The fungal mat showed little change in morphology by visual inspection afterwards except slight extension of some parts into the liquid culture media. The bottom of the mat remained yellowish white. The hyphae appeared septate under the light microscope as shown in Fig S1(b).

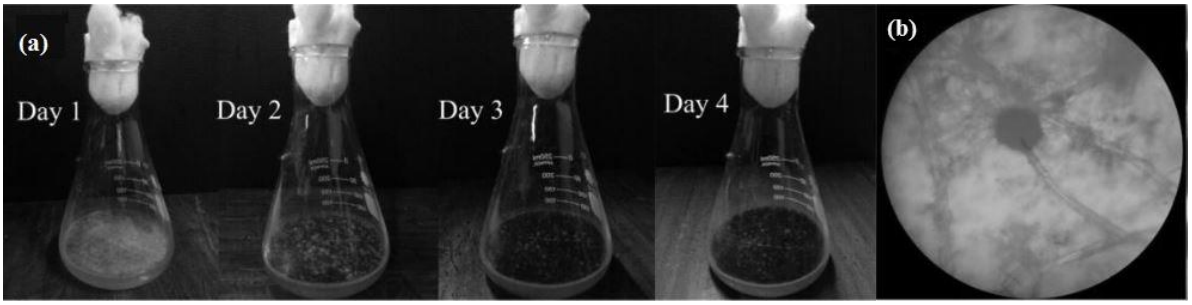

Fig S1 (a) Gradual change in morphology of *Aspergillus sp.*, (b) *Aspergillus sp.* under light microscope

S6. Growth of *S. cerevisiae* from commercial yeast suspensions

Sporulated yeast cells (*Saccharomyces cerevisiae*) were identified under light microscope as shown in Fig S2. These photos were taken at Day 4 of the nutrition starvation condition in sodium acetate solution. They were identified as *S. cerevisiae* characterized by the morphological shape and buds on the cells.[2]

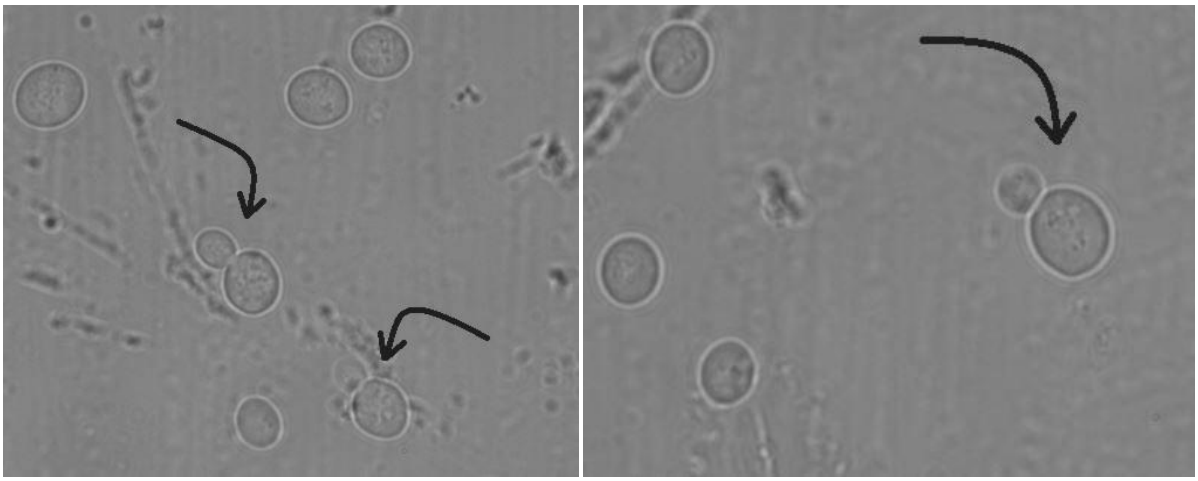

Fig S2 Sporulated *S. cerevisiae* under light microscope (100 X magnification)

S7. Isolation of *Staphylococcus aureus*

Staphylococcus aureus was identified by visual inspection from 1-day old MSAB (Mannitol Salt Agar Base) agar plates. The agar plate was shown in Fig S3. The part of the medium which displayed changed color (the original red color becomes bright yellow) is the part that contains *S. aureus* species. The yellow color represents production of ammonia and thus a pH lower than 7.0 that distinguishes *S. aureus* from other *Staphylococcus* species.

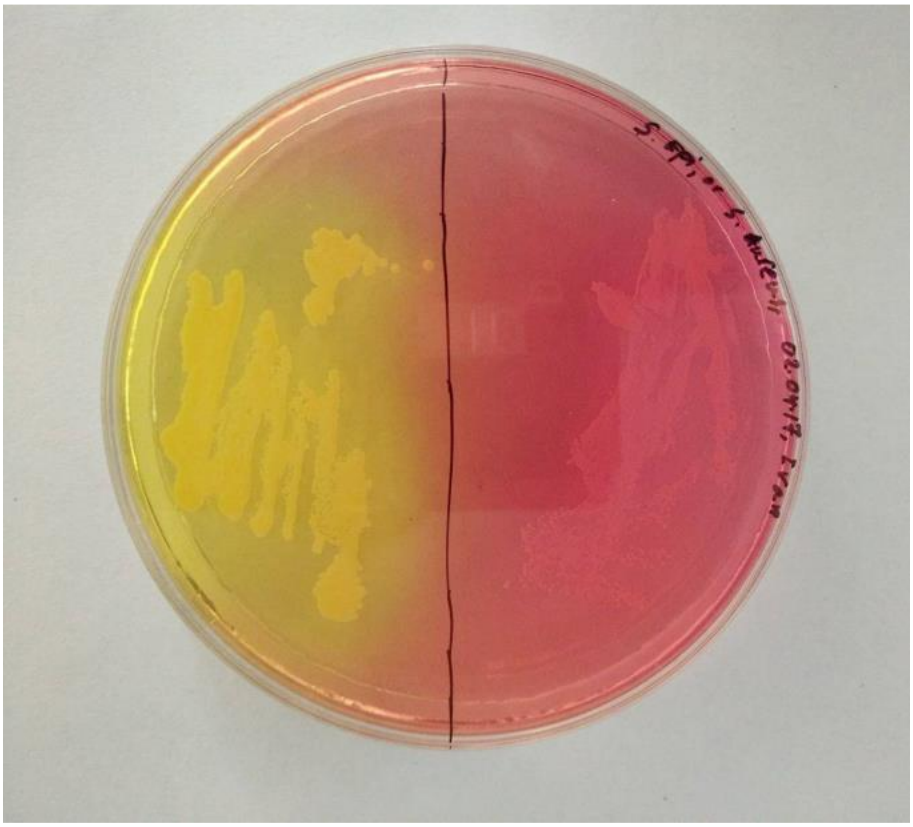

Fig S3 Isolation of *S. aureus* in MSAB

S8. Zones of inhibition of control and standard

Antimicrobial activity of the chitosan solution was observed through zones of inhibition. Fig S4 shows zone of inhibition of 1% acetic acid (control) and 1 g/L Tetracycline (standard). The measurable parameter that is directly linked to the extent of antibacterial activity is given by the diameter of the clear zones observed after a 24-hour incubation period. The larger the diameter of the zone of inhibition, the more potent is the antimicrobial action against the test pathogen, in this case, *Staphylococcus aureus.*

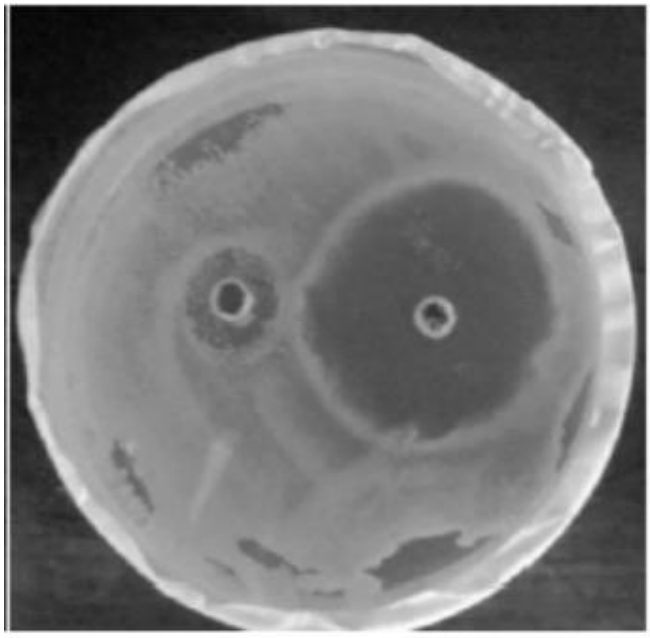

Fig S4 Zone of inhibition shown by 1% acetic acid (control) and 1 g/L Tetracycline (standard)