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Screening of chitin deacetylase producing microbes from marine source using a novel receptor on agar plate

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ABSTRACT

Chitosan is a deacetylated form of naturally occurring polymer; chitin. On an industrial scale, the deacetylation of chitin to chitosan is performed using harsh chemicals like sodium hydroxide. This not only adds to the environmental pollution but the product is also random in terms of its deacetylation. This shortcoming can be addressed by using enzymes like chitin deacetylase (CDA). The screening of these organisms would require a reliable, fast and sensitive screening method.

The deacetylation of chitin into chitosan, releases acetate as the byproduct of the reaction. A receptor which specifically binds to the acetate ion was synthesized chemically. The receptor upon binding with the acetate ion emitted a fluorescence which could be viewed using the gel documentation unit. The receptor was optimized for the screening of CDA producing microbes with the positive fungal control as *Penicillium* sp. and bacterial control as *Bacillus megaterium*. A parallel study with the 4-Nitroacetanilide, the reported screening indicator for CDA was performed. The results obtained with the receptor in the present study were concordant with the 4-Nitroacetanilide. Upon standardization, the protocol was extended for the screening of CDA producing microbes from the marine crustacean dumped soil and water samples. The CDA activity of these microbes was further confirmed using spectrophotometric MBTH assay.

This is the first report using this receptor for the screening of CDA producers. The method is not only sensitive but also reproducible and can be extended for a high throughput screening of CDA producers.

1. Introduction

Chitin found majorly in the marine organisms is the second most abundant biopolymer in nature. In-spite of its high abundance, it has limited application due to its high crystallinity [1]. To cater to this limitation, chitin is deacetylated to yield chitosan, a polymer with improved solubility and thus better commercial applications. This conversion which involves deacetylation can be performed either enzymatically or chemically. On an industrial scale, chemical deacetylation is practiced, which results in random removal of the acetate in chitosan. In addition, the usage of the chemicals results in a lot of environmental pollution due to the multitude of steps involved in the process [2]. The enzymatic route of converting chitin to chitosan makes use of chitin deacetylases (CDA) which hydrolyzes the acetamido group in the *N*-acetyl-D-glucosamine residues of chitin, producing the D-glucosamine residues of chitosan and releasing acetate. The reaction results in patterned deacetylation in chitosan [3,4]. It has been reported that every enzyme would have a distinct pattern of removal of the acetate group this creating a different pattern [3]. Thus the screening and isolation of CDA becomes pertinent. With the plethora of organisms available in the marine sample, the first step to develop a method for a fast screening is important. The present available method using p-nitroacetaanilide is time consuming with results not reproducible at times. The other method used for detection and quantification are based on using 3-methyl-2- benzothiazoninone hydrazone (MBTH) or calcofluor assay [4]. The selection process however becomes tedious and time consuming which ultimately reduces the rate at which novel CDAs would be discovered. As chitosan has tremendous industrial and medical applications, hence investing in a screening method would be worthwhile. This would also boost research in identification of novel CDA resulting in better patterns of deacetylation in chitosan. Hence in the present study we have tried developing a screening method based on the acetate that is released in as a by-product of the deacetylation reaction. Recently, Banerji and coworkers have reported a selective detection of acetate ion supported by NMR and DFT studies [5]. Researchers have so far reported probes to monitor endogenous metal ions and pH in live cells and organisms [6,7]. In the present study, in pursuit of specific and selective screening method for CDA producing organisms, we have developed a new series of highly selective

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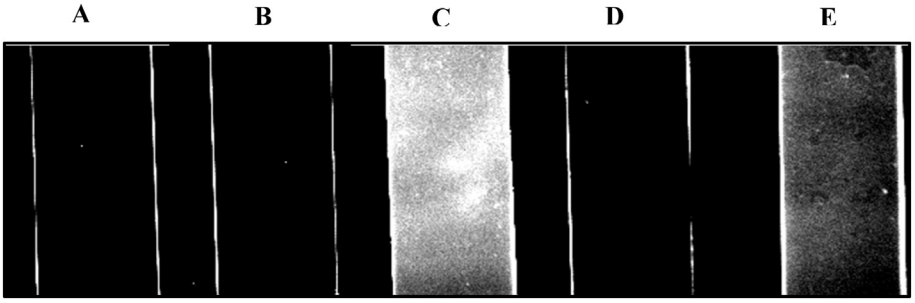

Fig. 1. Binding assay efficiency of receptor on solid surface. Panel A: chitin, Panel B: Chitosan, Panel C: Glacial acetic acid, Panel D: water, Panel E: LB media. The receptor upon interacting with acetate emits fluorescence as can be visualized in Panel C.

hydrazine based electro-optical receptor specific for the acetate ions based on fluorescence [8].

2. Methods and material

2.1. Materials

All chemicals and analytical grade reagents were used as bought without any further purification unless otherwise mentioned. Thin layer chromatography was performed using Merck TLC Silica Gel F254 plates. Melting point was measured on Stuart SMP3 melting-point apparatus in open capillaries. Infrared spectra were recorded on Bruker alpha FTIR spectrometer. ^{1}H NMR was performed using Bruker-400 AV-400 spectrometer. Chemical shift values are reported in ppm scale (in DMSO-d_6 with Tetramethylsilane as internal standard). UV–Vis experiments were performed on analytik jena Specord S600 spectrophotometer in standard 3.0 mL quartz cuvette having 1 cm path length.

2.2. Preparation of the receptor

The receptor was synthesized and characterized using standard spectroscopic techniques [8].

2.3. Preparation of colloidal chitin agar plates

Colloidal chitin was prepared according to the method described by Rodriguez et al. [9] with a few modifications. Briefly, 2.5 g of α-chitin was dissolved in 100 mL of cold HCl, stirred for 45 min at 37 °C. One liter of distilled water was added to the dissolved chitin. The suspension was allowed to mix at 4 °C for a half hour, and later allowed to stand overnight at the same temperature. Solids were separated by vacuum filtration and filtrate was discarded. The colloidal chitin residue was further washed four times with distilled water, and the pH was raised to neutral using 0.2 N NaOH. The residual NaOH was later removed by water washes. This colloidal chitin was stored at 4 °C for further use. The stock solution of colloidal chitin was maintained at 10% (w/v). The colloidal chitin agar plates were prepared with the final concentrations of: $NaNO_3$ – 2 g/L K_2HPO_4 – 1 g/L, KH_2PO_4 – 1 g/L, $MgSO_4$ – 0.5 g/L colloidal chitin – 1% (w/v), agar – 2.5% (w/v) and pH was maintained at 7 ± 1.

2.4. Preparation of receptor agar plates for fluorescence measurement

A 5% (w/v) receptor stock solution was prepared in 100% ethanol. To this solution, 1% (w/v) DMSO was added to dissolve receptor [8] completely for use. The solution was prepared fresh for better results. The receptor solution (20 μL) was spread on the colloidal chitin agar plates (20 mL) and allowed to dry for 30 min in the laminar air flow chamber. Receptor containing glass slides were also made with different chemicals such as chitosan, glucosamine, *N*-acetyl glucosamine and acetate to study selective binding of receptor to these chemicals. The receptor binding assay on the glass slide was done by spreading the receptor solution with different reaction components and viewing the fluorescence.

2.5. Inoculation of Penicillium glaucum

Penicillium sp. was procured from Microbial Type Culture Collection (MTCC 5108), Institute of Microbial Technology, Chandigarh, India. The culture was revived as per the specifications. The culture was grown at 30 °C for all the receptor optimization experiments. The initial experiment with the organism was performed on the para–nitroacetanilide (PNAA) LB agar, an extrapolated method of CDA detection in liquid media as reported by Yang et al. [10] for the detection of chitin deacetylase activity.

2.6. Inoculation of Bacillus megaterium

B. megaterium was procured from Microbial Type Culture Collection (MTCC 14945), Institute of Microbial Technology, Chandigarh, India. The revival of the culture was done on the colloidal chitin agar plate for 2 days at 30 °C. A starter culture was made of 5 mL of colloidal chitin broth (Composition same as the glycol chitin containing agar plates). This culture was incubated overnight at 30 °C and 150 rpm. The culture

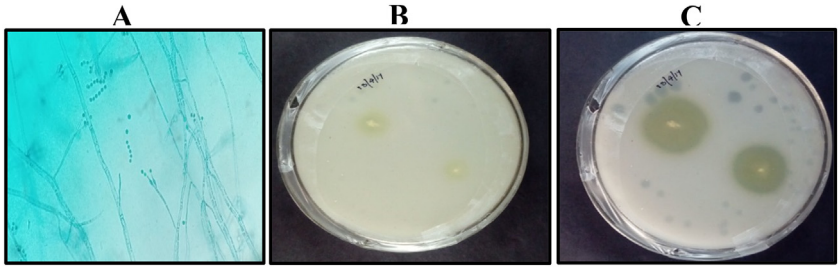

Fig. 2. *Penicillium* sp. screened on PNAA spread colloidal chitin plates. Panel A: Microscopic analysis of Penicillium sp. using lactophenol staining. Panel B: CDA activity depicted as a yellow coloured zone around the fungus as seen after 5 days post inoculation. Panel C: CDA activity 7 days post inoculation.

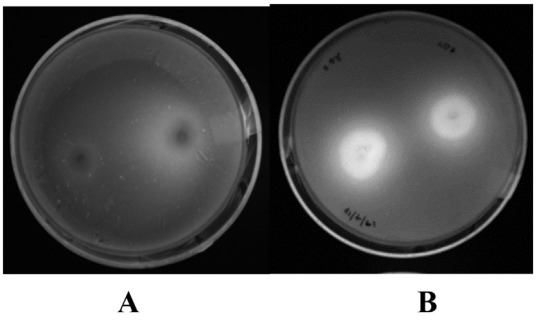

Fig. 3. Optimization of fluorescence intensity with the inclusion of DMSO in the receptor solution. A: Plate with 0.1%receptor dissolved in absolute alcohol. B: Plate with receptor dissolved in absolute alcohol with 1% DMSO. *Penicillium* sp. was used for the test.

was used as inoculum (1%) for 10 mL of colloidal chitin containing medium. This culture was incubated at 30 °C and 150 rpm for 12 h. An aliquot of 50 μL of the culture was spread plated on receptor agar plates. The plates were incubated at 30 °C and the resultant chitin deacetylase activity was observed with the gel-doc imager (Bioscreen, USA) at 365 nm. The plates were observed for a period of 7 days at an interval of 24 h. Blank plates were prepared without addition of any culture broth. A positive control of the transformed *E. coli* Rosetta pLysS cells containing chitin deacetylase gene was taken [11]. The plasmid containing chitin deacetylase was a kind gift from the Professor Moerschbacher, WWU Muenster.

2.7. Enzymatic assay for detection of chitin deacetylase activity using MBTH

This assay is based on the deamination of glucosamine (monomer of chitosan) to aldehyde with nitrous acid followed by colour development using 3-methyl-2-benzothiazoline hydrazone (MBTH). The enzyme chitin acetylase converts chitin into chitosan. The chitosan thus produced is detected using MBTH assay. 250 μL of glycol chitin (1 mg/mL) and 250 μL of Tris-HCl buffer (pH 6.5) consisted of the assay mixture and 100 μL of the culture broth was added to the assay mixture as a source of the enzyme [12]. This mixture was incubated at 37 °C with shaking for 1 h. Then 500 μL of 5% $KHSO_4$ was added to the samples to stop the reaction and kept for 5 min. Then 500 μL of 5% $NaNO_2$ was added to the samples. Addition of sodium nitrite was followed by 6 h of incubation in the fume hood to release all the NO_2 gas. This was then followed by the addition of 500 μL of ammonium sulphamate (12% w/v) and 500 μL of 0.5% after 5 min of incubation and kept for 1 h incubation. Finally 500 μL of 0.5% $FeCl_3$ in 0.1 N HCl was added. This was incubated for 1 h and the absorbance measured at 656 nm. The buffer was used as a reference without the enzyme or substrate. Enzyme blank and substrate blanks were also used. One Unit of the enzyme is defined as the activity which released 1 μmol of glucosamine from the glycol chitin per minute. The enzyme activity of the sample was calculated accordingly.

The established method was further extrapolated for screening of microorganisms from crustacean soil dump. One gram of the soil sample was taken and a dilution series made using water as the solvent. 100 μL of each dilution was later spread to the colloidal chitin agar plates and incubated at 37 °C for the appearance of the colonies. The colonies

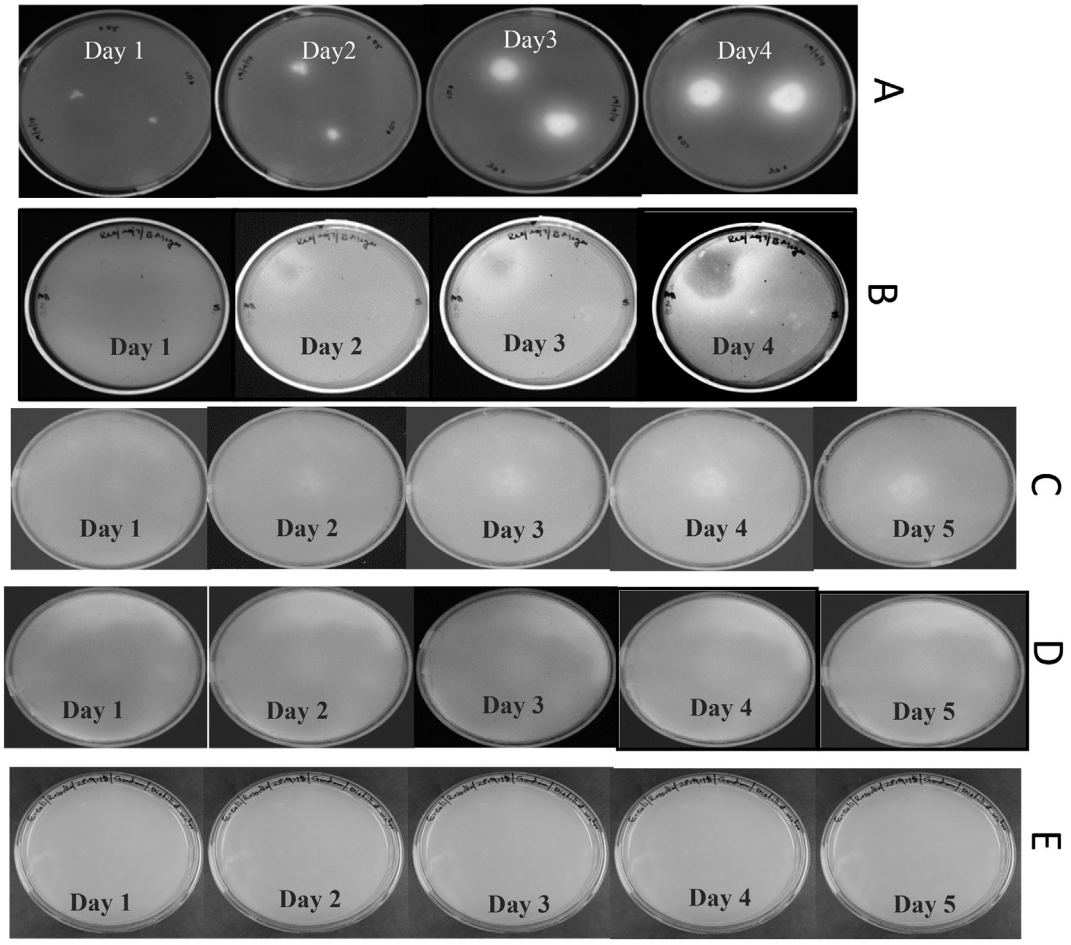

Fig. 4. Detection of CDA activity on the receptor based colloidal chitin agar plate. A: *Penicillium* sp. B: *Bacillus megaterium*, C: *E. coli* host cells with CDA gene induced, D: *E. coli* host cells with un-induced CDA gene, E: Empty *E. coli* host cells.

were purified by repeated streaking for four times and later point inoculated on the receptor plate. The CDA activity was further quantitated by 3-methyl-2-benzothiazolone hydrazine (MBTH) assay with 16 hour sample.

3. Results

The chitin deacetylase catalyzes deacetylation of chitin resulting in chitosan and acetate ions. In the present study, the specific binding of receptor with the acetate has been exploited [8]. The receptor studies conducted by the authors in the liquid surface depicted a chromogenic shift with the acetate binding. However, this binding resulted in fluorescence instead of a chromogenic shift on a solid surface (data not shown). To check for the binding specificity of the receptor; chitin, chitosan, glacial acetic acid, water and LB agar were spread on the glass plate along with the receptor (Fig. 1). Fluorescence was observed only with the glacial acetic acid. The slide with LB media spread displayed translucency due to the peptone and yeast extract ingredients present in the media. The initial optimization experiments with the receptor were done using *Penicillium* sp. as the model organism. An initial confirmatory experiment was conducted using *Penicillium* sp. on the PNAA LB agar plates (Fig. 2) where a chromogenic shift of the activity zone around the fungus was observed after a period of five days. The activity was visualized as a yellow coloured zone around the fungus. A pronounced activity zone was observed after a period of 7 days post inoculation. In parallel, experiments with the receptor was carried on the colloidal chitin plate (Fig. 3). In the receptor plate (with 0.1% receptor concentration) a slight fluorescence was observed after a period of 2 days which intensified till 4 days and then saturated. To extend the detection methodology to bacteria, *Bacillus megaterium* and *E. coli* (as the transformed host carrying plasmid with CDA gene) were made (Fig. 4). A slight fluorescence was observed with *B. megaterium* from the second day post inoculation. In the case of *E. coli* clones, fluorescence was observed when the clones were induced with IPTG (Fig. 4) while no activity and hence fluorescence was visible in the un-induced clone. The empty *E. coli* host was not able to grow on the colloidal chitin plates even after a period of five days. The optimized receptor conditions were further used for the screening of CDA producers from crustacean soil dump. All of the ten isolates which were purified were point inoculated on the receptor plates. Out of ten isolates which were inoculated, four gave fluorescence (Fig. 5). The CDA activity of these isolates was later quantified with the MBTH Assay (Fig. 5). The highest activity was obtained as 0.84 U/mL in S7 followed by S9 at 0.75 U/mL. The overall CDA activities ranged between 0.25 U/mL and 0.84 U/when the isolates were cultured in the colloidal chitin medium. The activities however fell to a range of 0.21 U/mL–0.28 U/mL when cultured in nutrient broth.

4. Discussion

One of the bottlenecks in the isolation of CDA producers is the agar based screening method. The available method of screening involves the use of PNAA, a colourless compound which upon the action of CDA gets converted to yellow coloured p-nitro anilide (PNA) [10]. This method though a bench mark in the liquid media, suffers from limitations of reproducibility and sensitivity yielding false positives and negatives when extrapolated on the solid media. In addition the screening protocol is time consuming. This limitation was addressed by the use of a receptor [7], which has been reported to specifically bind to acetate. We have attempted to screen for CDA producers based on this specific interaction. The receptor, a chromogenic indicator of acetate in the liquid state surface did not display any change in colour on the solid surface (results not shown), but instead emitted fluorescence. The

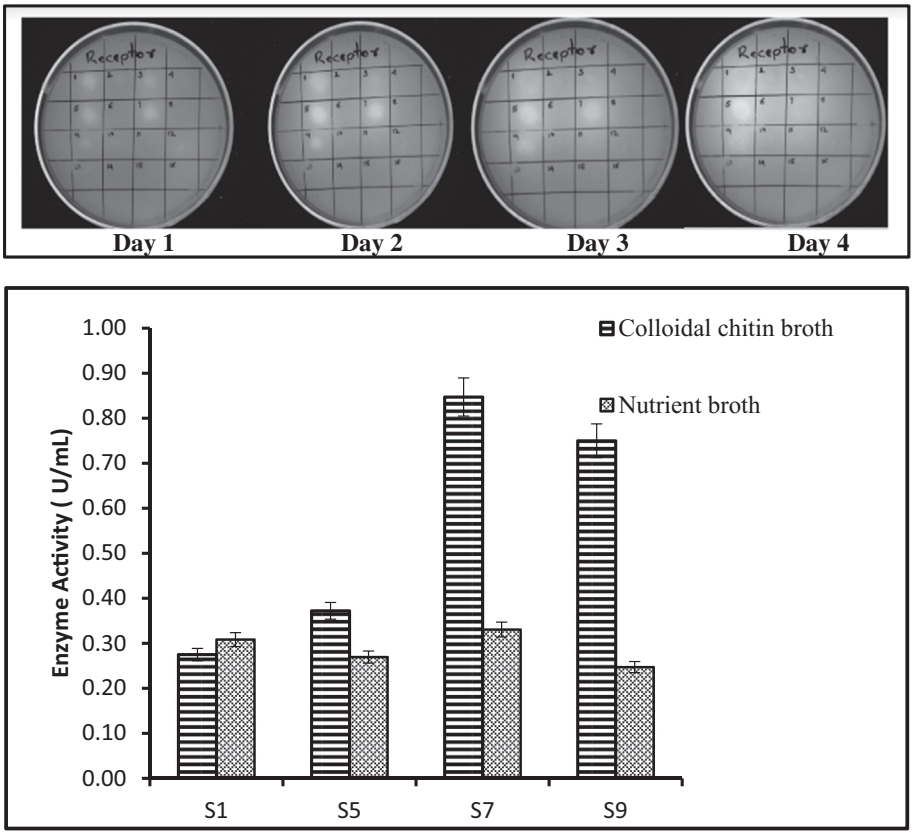

Fig. 5. Screening of crustacean soil dump for CDA producers. The receptor plates were point inoculated with the purified microorganisms from the soil dump for the microbes producing CDA. The plate was divided into a grid of 4 × 4. The isolates emitting fluorescence were subjected to MBTH assay (shown in the lower panel). The activity was measured both in the colloidal chitin broth and nutrient broth to evaluate the production media for maximal CDA production.

cross reactivity of the receptor with other components of the reaction mixture was also tested. The test proved that the receptor emits fluorescence upon binding with acetate and gave a negative result wit chitin, chitosan, and water and LB media solutions. We wanted to confirm the reaction conditions so acetic acid was used at a dilution of 40%. This was performed to make sure that the acetic acid is ionized to its anionic form. The receptor solution reported for the liquid medium was dissolved in 10% DMSO [8], but as we were initiating microbial screening, the DMSO inclusion in the solution was omitted. Thus the receptor solution prepared was prepared in 100% ethanol but it gave a very weak fluorescence. The main reason for this was the incomplete dissolution of the receptor in absolute alcohol. Hence in the consecutive experiments, DMSO was included in the receptor solution at a final concentration of 1%. This concentration did not prove to be inimical to the microbes. The inclusion had improved the fluorescence as measured at 365 nm excitation. The receptor based method was first tested with *Penicillium* sp., a known CDA producer [12,13]. *Penicillium* sp. also demonstrated chitin deacetylase activity using PNAA as the marker substrate but the results were visible after a span of 5 days. The same organisms when inoculated on the receptor plate gave detectable activity within two days. The same test was repeated with *B. megaterium*, which has a weak chitin deacetylase activity [14,15]. Its activity could not be detected with the PNAA plate but with the assay method developed in the present study, a weak fluorescence was visible from the second day post inoculation. The fluorescence intensity was however lower than the *Penicillium* sp. In another set-up, the transformed *E. coli* Rosetta pLysS clone was used. Two sets of experiments were made, in the first set, the clone was induced with 1 mM IPTG *in-situ* while an uninduced control was kept in another plate. There was growth observed in both the plates. It is known that there are chances of leaky expression in *E. coli* with T7 based promoters [16]. We had used pET22 b vector which has T7 lac promoter, hence this could be a reason that the uninduced clone grew on colloidal chitin plate but gave no detectable activity. To test this hypothesis, the host (*E. coli* Rosetta pLysS) was plated on the receptor colloidal chitin plate which had no growth even after 5 days post inoculation. In an attempt to test the receptor assay for environmental samples, the assay was extrapolated to screen the crustacean soil dump for CDA producers. The plate assay yielded four positive isolates. These isolates were further tested and quantified with MBTH assay. The results of the two tests were concurrent with the isolate S7 giving the highest activity of 0.84 U/mL followed by S9 at 0.74 U/mL.

In conclusion, a sensitive and reproducible assay method for screening has been developed which can be used for high throughput identification of CDA producers from novel environmental sources.

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