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ISOLATION AND SCREENING OF EXTRACELLULAR CARBOXYMETHYLCELLULASE PRODUCING MARINE FUNGI FROM AUROVILLE BEACH, PONDICHERRY

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Abstract:

Cellulose is the most abundant renewable natural product in the biosphere. The proportion of cellulose in plant tissues ranges from 20 to 45% of their dry cell weight and to almost over 90% in cotton fibers. Totally 38 different fungal isolates were isolated two different places in Auroville beach, Pondicherry in PDA from enriched sample. Among the 38 isolates 15 isolates showed the CMCase activity in CMC broth. The 38 isolates were grown for 7 days. Based on microscopic characteristics the potential isolates S7, S6, S11 identified they belongs to Penicillium, Aspergillus, Fusarium, generas. The enzyme CMCase production was analyzed by checking both intracellular and extracellular CMCase activity of the isolates. The isolate S6 was selected for progress of the work based on its enzyme activity and production time. It was conformed that the enzyme produced by the isolate was extracellular. Immobilization of enzyme was performed the purified enzyme was showing 30% activity in the column at the flow rate of 1ml for 1minate.

Keywords: *cellulose, carboxymethyl cellulase, fungi, extracellular enzyme, Immobilization*

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I.INTRODUCTION:

Cellulose is the most abundant polysaccharide that has tremendous economic importance around the globe. It is a linear, unbranched homopolysaccharide consisting of glucose subunit joined by β 1-4 glycosidic linkage. It is a primary structural component of the plant cell wall accounting for over half of the carbon in the biosphere [1,2]. Cellulose is the most common abundant, renewable biopolymer on earth and domestic waste materials from agriculture representing about 1.5 x 10^{12} tons of the total annual biomass production through photosynthesis in the topics [3,4]. Cellulose is considered as one of the most important sources of carbon on this planet and its annual biosynthesis by both land plants and marine occurs at a rate of 0.85×10^{11} tons per annum [5]. Cellulase degradation and its subsequent utilizations are important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest [4]. There has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency (Subramaniyan and Prema, 2000).

Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization [6]. Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation [7]. Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for bio-polishing of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness [8]. Application of enzymes in textile, food, detergent, leather and paper industries demands identification of highly stable enzymes active at extreme pH and temperature [9]. Cellulase is used in the fermentation of biomass into biofuels [10], fiber modification and they are even used for pharmaceutical applications. The aim of this study was to isolation, screening and characterization of cellulase producing fungi from marine environment.

2.MATERIALS AND METHODS:

2.1. sample collection

The sample was collected from near to Auroville beach, Pondicherry in the condition of high moisture, wood degraded drift soil sample. The sample was collected in the sterile zip like bag. The bag was kept in the ice box and stored in refrigerator.

2.2. Sample enrichment

The sample enrichment was done by following modified mandel's method and media .The media contain respected ratio of following components, peptone -(0.1%), urea -(0.03%), $MnSO_4.H_2O$ –(0.0023%), $ZnSO_4.7H_2O$ -(0.0014%), $(NH_4)SO_4$ -(0.14%), $MgSO_4.H_20$ -(0.0016%), $FeSO_4.7H_2O$ -(0.05%), $CaCl_2.6H_2O$ –(0.0029%), KH_2PO_4 –(0.2%). 1g of soil sample was carefully transferred into the mandel's media, then the media was incubated at 28°C for 7 days with shaking condition.

2.3. Isolation of fungus from enriched sample

The enriched sample was serially diluted using with physiological saline and diluted sample were spared on sterile potato dextrose agar medium, the media is generalized media for fungi, it contain following components, Potato infusion (200g/l), Dextrose (20g/l), Agar powder (20g/l) and the media was incubated at 28°C for 5 days.

2.4. CMC Agar media

The fungal isolates which are grown on Potato Dextrose Agar were individually inoculated on CMC (carboxyl methyl cellulose) Agar media contain following components for 500ml media $NH_4H_2PO_4$ -0.5g, KCl -0.1g, $MgSO_4.7H_2O$ -0.5g, Yeast extract -0.5g, Carboxymethylcellulose -13g and Agar -1.5 g. The media was incubated at 28°C for 5 days. After incubation, the zone was identified around the culture by treating with Congo red and NaCl.

2.5. Measurement of enzyme activity

Enzyme activity was assayed using a modified method described by *Wood and Bhat* with some modifications. 0.2 ml of culture filtrate was added to 0.8 ml of 1% carboxymethyl cellulose prepared in 0.1M sodium acetate buffer (pH 5) in a test tube and incubated at 50°C for 60 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in a water bath at 100°C for 15 min. Absorbance was recorded at 540nm against the blank (0.1 M phosphate buffer).One unit of enzyme activity was defined as the amount of enzyme that released 1 µM of glucose per min.

2.4. Estimation of Reducing Sugars Content

Reducing sugars analysis was conducted by using 1 ml of sample which was added to 3 ml of DNS and boiled for 15 min. The absorbance was recorded at 540nm using a spectrophotometer against the blank of distilled water.

2.5. Optimization of Cultivation Medium

2.5.1. Effect of nitrogen source on CMCase production

Nitrogen source in the minimal medium was substituted with various nitrogen sources at 1.0% (w/v): beef extract, yeast extract, ammonium nitrate, peptone and ammonium chloride.

2.5.2. Effect of Temperature and pH on CMCase production

The effect of pH on the CMCase production was determined by measuring the enzyme activity using different buffers at 30°C. Sodium acetate (pH 4.0 to 5.5), Tris (pH 6.0 to 8.5) sodium carbonate (pH 9.0) and glycine-NaOH (pH 10.5 to 12.5) buffers were used at different pH conditions. The effect of temperature on the reaction rate was determined by performing the standard reaction at different temperatures in the range of 20-35°C. The enzyme activity and specific activity was determined.

2.5.3. Effect of Metal ions on CMCase production

The effect of different metal ions ($ZnCl_2$, $MgCl_2$, $CaCl_2$ and $FeSO_4$) on the CMCase production was determined by using the different concentrations (0.1, 2.5 and 5mM) of metal ions. The culture was incubated at 30°C for 3 days at 100 rpm on an orbital shaker. The biomass and emulsification activity was determined.

2.6. Immobilization of enzyme

Calcium alginate is a water-insoluble, gelatinous, cream colored substance that can be created through the addition of aqueous calcium chloride to aqueous sodium alginate. Adding artificial flavors and colors creates a more tasty edible slime. Calcium alginate is used for entrapment of enzymes and forming artificial seeds in plant tissue culture. 25cm column was prepared with beads

3. Results and Discussion

3.1. Sample collection and processing

The sample was collected at two different places in Auroville beach (Fig.1), Pondicherry. To isolate more specific cellulolytic fungus the sample was collected at the places of wood degrading and moisture condition. To avoid the other environment microbe's contamination, sample was collected with sterile zip like big and stored in refrigerator.

Fig.1 Sample collection

3.2. Sample enrichment

The sample enrichment was done, to increase the chance of isolating more specific cellulose producing fungi. The method followed was Modified Mandel's Method; the media contain 1% wood chips (Fig.2) so it provides support to the growth of cellulolytic microbes

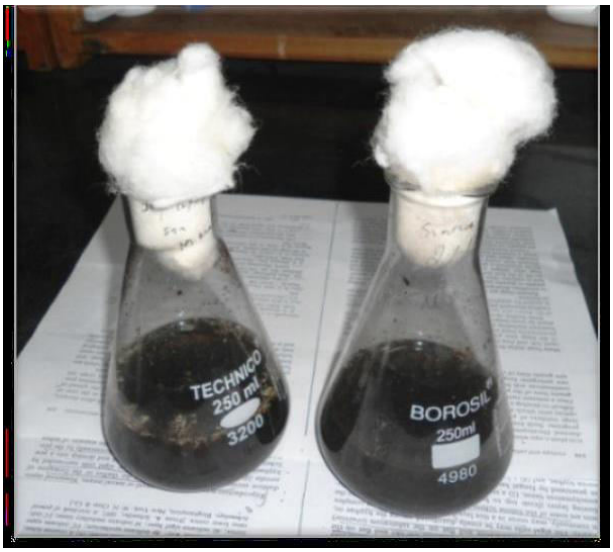


Fig.2 Sample enrichment technique

3.3. Isolation of fungus from enriched sample

38 different fungal isolates were isolated in PDA from enriched sample (Fig.3). The isolated fungal isolates are individually grown in PDA slants and stored in 4°C. Totally 21 fungal strains were isolated from to different soil samples, i.e. 12 isolates from paper industry soil sample and 9 isolates from bami soil sample (Lekh Ram *et al.,* 2014). The Fungi grown on the selective media supported the growth of the fungi by using cellulose as the carbon source (Khalid *et al.,* 2006).

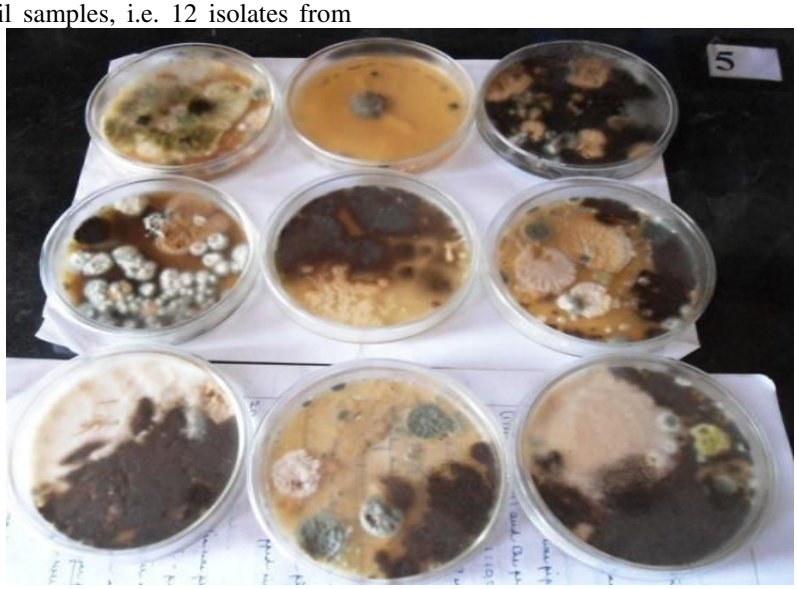


Fig.3 Fungal Isolates in PDA

3.4. Screening of cellulolytic enzyme producing isolates

Enzyme activity was assayed using a modified method described by Wood and Bhat with some modifications. 0.2 ml of culture filtrate was added to 0.8 ml of 1% carboxymethyl cellulose prepared in 0.1M sodium acetate buffer (pH 5) in a test tube and incubated at 50°C for 60 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in a water bath at 100 0C for 15 min. Absorbance was recorded at 540nm against the blank (0.1 M Sodium acetate buffer).One unit of enzyme activity was defined as the amount of enzyme that released 1µM of glucose per min.

Among the 38 isolates 15 isolates showed the CMCase activity in CMC broth (Fig.4). The 38

isolates were grown for 7 days. Enzyme activity was checked after 7 days and following results were noted. Cellulase activity of the enzyme was measured by cellulase assay. Cellulase Assay was done by DNS method (3, 5-dinitrosalicylic acid) and the activity of the enzyme was expressed in mg/ml/min. It was calculated by the following formula: Enzyme activity = amount of glucose liberated/mg protein/30minutes.In the previous studies made on cellulase by Lone M.A. *et al.* (2012), the cellulase activity of *A. niger mtcc872* was 59.25mg glucose liberated/mg protein/30minutes. But in our work the enzyme activity of the PISS-3 isolate was found to be 20mg glucose liberated/mg protein/30min at 32°C [11].

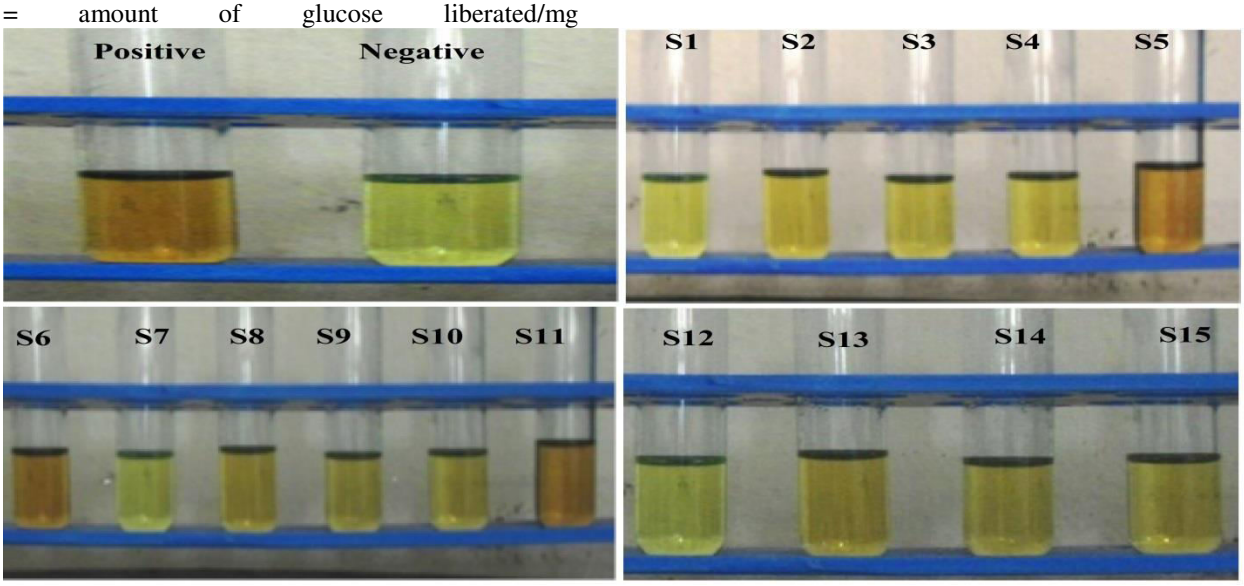


Fig.4a Enzyme assay for isolate

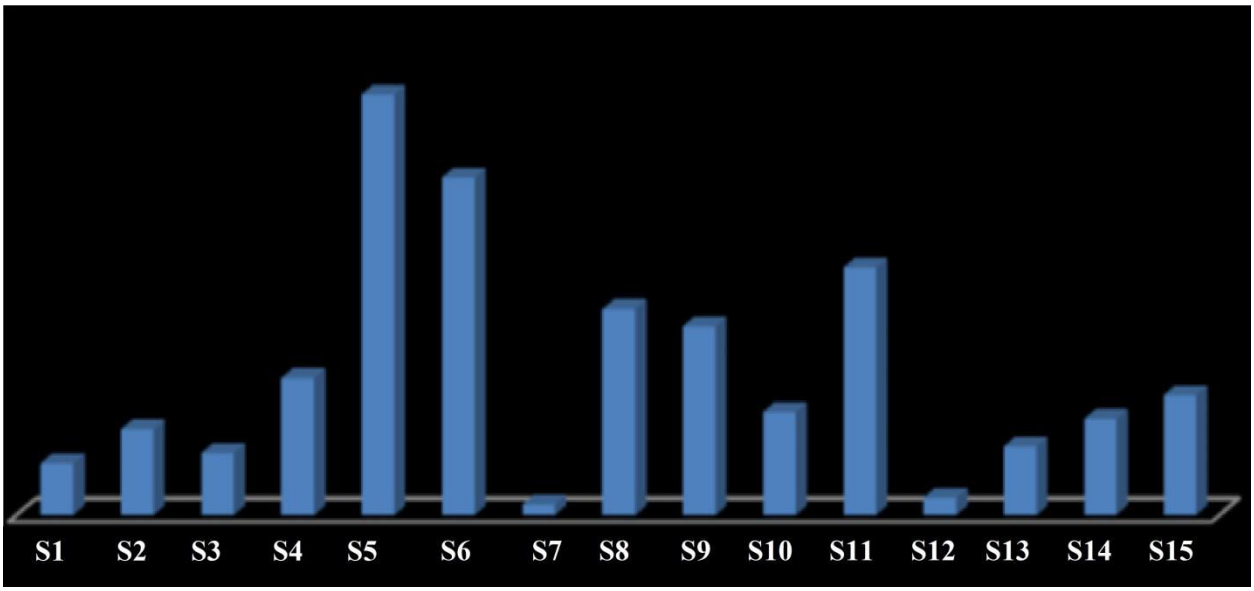


Fig.4b CMCase production of Marine Isolates after 7 days incubation

3.5. Plate assay for CMCase

The 15 isolates were inoculated in CMC media, and were incubated at 28°C for 5 days. After incubation, the zone was identified around the culture by treating with Congo red and NaCl. Among the 15 marine isolates 3 isolates (S5, S6, S1 1) showed the zone of hydrolysis (Fig.5). Efficient cellulase producing fungi isolates were finally selected based on the zone of the clearing around the fungi on carboxyl methyl cellulase agar (CMC agar) plates [12,13]. The appearance of the clear zone around the colony when the Congo red solution was added [14]

was strong evidence that the fungi produced cellulase in order to degrade cellulose. Out of 21 fungal isolates, only 4 isolate produced zones of hydrolysis in CMC agar plates within 3 days and results were represented [15].

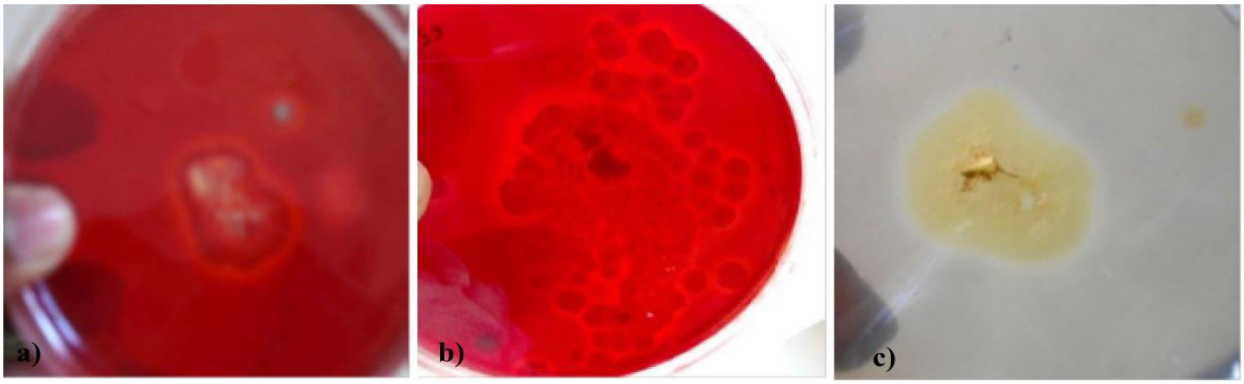


Fig.5 Zone of CMC hydrolysis (a&b - Congo red staining and c – Iodine staining)

3.6. Microscopic Observation

The CMCase producing (S7, S6, S11) were observed under light microscopy after staining with lactophenol cotton blue. The morphology of isolate S6 was, septate mycelia, branched conidiophore, a bunch of sterigmata on the conidiophores, conidiophores were arranged like linear chain and the conidiophore size were small. The morphology of isolate S7 was, aseptate mycelia ending with sphere shape, it contains bottle shaped sterigmata and small conidiophores were arranged on the sterigmata. The morphology of S11 was, it has mycelia rarely septate, ends with sharp end, and some time little large conidiophores were observed.

Based on microscopic characteristics the isolatesS7, S6, S11 identified they belongs to *Pencillium, Aspergillus, Fusarium,* generas (Fig.6).

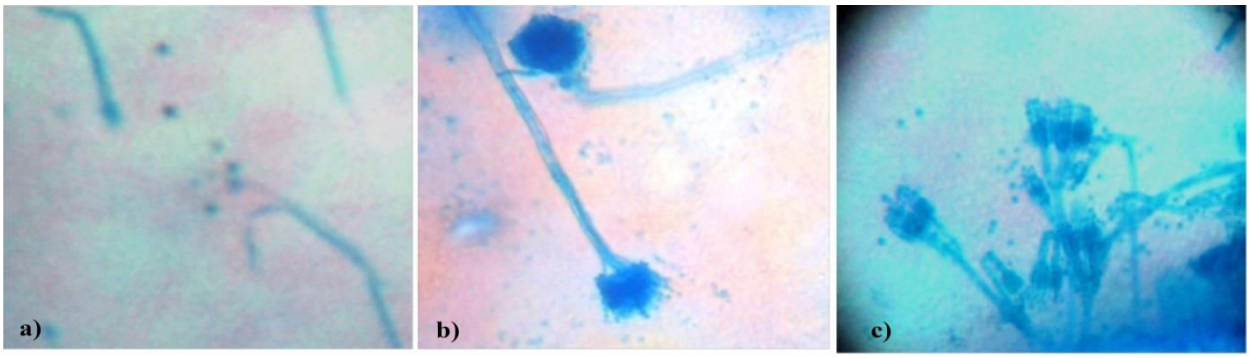


Fig.6 Light microscopic images for isolates, (a)S7, (b)S6, (c)S11

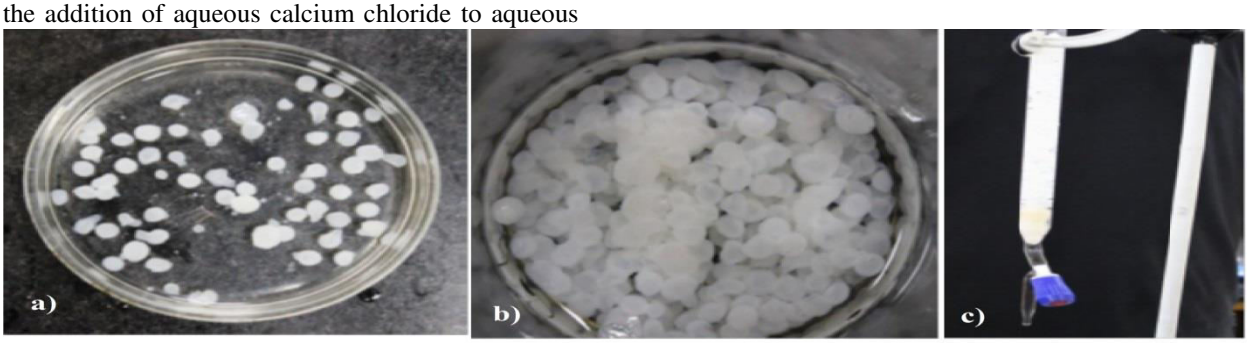
3.7. Media Optimization for enzyme production

The enzyme CMCase production was analysed by checking both intracellular and extracellular CMCase activity of the isolates. The isolate S6 was selected for progress of the work based on its enzyme activity and production time. It was conformed that the enzyme produced by the isolate was extracellular. Six different nitrogen sources were tested for CMCase activity. Production media supplemented with peptone showed maximum CMCase activity (130.5 U/ml) and yeast extract showed (125 U/ml) enzyme activity. Other nitrogen sources such as urea, $NaNO_3$, NH_4Cl and beef extract also showed CMCase activity. The maximum CMCase activity was observed at 28^oC. CMCase activity was inhibited at temperature 20 and 40°C, at temperature 20^oC activitie was around 102.3 U/ml and at 40^oC CMCase production was 76.3 U/ml. At 28^oC maximum CMCase activity 122.6 U/ml was observed. Among the various pH tested maximum CMCase activity was observed at pH 5.0 is 122.3 U/ml. when the pH was altered activity was decreased. This may be due to inactivation of enzyme. Different metal ions were added in the production media to test their production of CMCase. Maximum CMCase activity was observed in production media which was supplemented with 5mM $MgSO_4$ 124.2 U/ml. A drastically drop was noticed for other metal ions $MgSO_4$ has a little inhibitory effect on CMCase activity. The concentration of $MgSO_4$ was further

increased, their no observable change in CMCase activity (Abreham *et al.,* 2015).

3.8. Immobilization of enzyme

Calcium alginate is a water-insoluble, gelatinous, cream colored substance that can be created through the addition of aqueous calcium chloride to aqueous sodium alginate. Calcium alginate is used for entrapment of enzymes and forming artificial seeds in plant tissue culture. Immobilization of enzyme was performed the purified enzyme was showing 30% activity in the column at the flow rate of 1ml for 1minute.



(A, B) Calcium alginate beads, (C) Calcium alginate beads in column

4. CONCLUSION:

In this study, 38 fungal isolates were isolated from Auroville beach, Pondicherry. Among the 38 isolates only 15 isolates were produce CMCase activity on CBM. Out of the 15 isolates only three efficient isolates namely: *Penicillium* (S7), *Aspergillus* (S6) and *Fusarium* (S11) were recovered. *Penicillium* sp showed maximum zone of hydrolysis followed by *Aspergillus* sp and *Fusarium* sp. In this study, the decaying wood chip was found to be a good source of cellulolytic fungi species. For application in industries, further characterization and optimization of the culture condition is needed to suite industrial application of this potential fungi species for cellulose bioconversion in industries.

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