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Isolation, Identification and Screening of Chitinase Producing Aspergillus Niger from Soil

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ABSTRACT

Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. Chitinase enzyme has received increased attention due to its wide range of biotechnological applications.In the present study an attempt was made to isolate organisms that would synthesize chitinaseChitinase may convert the biomass containing chitin into useful components (depolymerized).

This project aimed to isolation and identification of chitinase enzyme producing Fungi such as Aspergillus niger. In order to isolate chitinase from fungi, the possibility to obtain this enzyme from higher organisms such as animals, plants or insects are less. Since production of chitinase from higher organisms is a tedious process and also its purification is challenging.

Aspergillus niger was found to be a good chitinase producer among the five fungi isolated from different soil samples Punjab(India). Maximum production of chitinase was obtained when using 2% of Shrimp-shell powder as a sole carbon source in the fermentation medium. The high level of chitinase production was observed in the culture medium.

Keyword: chitinase, chitin, Aspergillus niger, isolation, screening

1. Introduction

Chitin is the major component of most fungal cell walls, that is usually combined with β-1 and 4-remaining N-acetyl D-glucosamine linked to glucosamine [8].

Most of the studies on Chitinase production have been carried out in liquid or submerged condition. However, some attempts have been done in more recent times on Solid-State- Fermentation (SSF). Mostly, presence of chitin in production medium is useful for the production [22].Among the various substrate, Colloidal chitin was found to best substrate for Chitinase production [23]. Addition of carbon sources other than chitin repressed the Chitinase production while supported the growth. In most cases, chitin concentration in the range of 1-1.5% was found to be suitable for chitinase production [26].

In most organs, chitin is regulated by the formation of bonds with other polymers such as glucan, protein; etc [21].Chitin substrate since to cellulose is the latter polymer [6].Marine squanders are one of the great wellsprings of Chitin, with more than 80,000 tons of chitins being produced every year from marine waste, collecting large amounts of marine waste has become a major source of dependence on the seafood processing industry. Hence, the generation of significant worth included items, for example, Chitin, Chitosan and its structures for profitable roles in various tendencies and important sectors of the farm are essential. Chitin is a natural product such as cellulose [20].

Chitin is usually associated with other compounds such as proteins, lipids, calcium carbonates and pigments, and is associated with the conversion of these chemical waste into useful chitin and the associated oligomers include proteins such as proteinuria, demineralization, or hydrolysis, previously with a strong acid base, which includes high costs, low performance and corrosion [26].

A possible alternative to this problem is the use of the chinolytic enzyme, chitinase, and B-N-acityl hexosaminidase. Chitin is highly resistant to chemical reactions and requires strong mineral acids to dissolve. It is necessary here that

absorbable enzyme of chitinase have a great secondary nutritional role for citrus sources of nitrogen and carbon [12].

Chitinase is found in various microorganisms (bacteria, fungi) and animals (snails, poppy skin, vertebrates, insects and beans) [17]but, the most suitable source and the best source of chitinase are microorganisms. The present-day communication trucks play a key role in the making of enzyme. Chitinase acts in the biosphere carbon cycle in protecting ecosystems [1].

Chitinase in microorganisms and vegetables with a variety of organism's plays impress like metabolism in growth hips, protection instruments because of pathogens, nutrition, parasitism and biological stress [9].Chitinase is an enzyme equipped for disintegrate unsolvable substrate to oligo molecules. Chitinase also acts and functions in the creating and making of single-cell protein (SCP) to animal for the separation protoplast of fungus, production of inactivated bioavailable phytopathogen and quitosaccharides, and destruction of chitin waste[1].

The production of inexpensive quinitolinic enzymes, solves environmental problems, still reduces the cost of producing quitogasacarid for the production of products such as sweets, and several growth factors and single cell protein [7].

2. Research Methodology

2.1 Substrate

Chitin solution as the substrate utilized for the creation of chitinase enzyme by chitinolytic fungus strains [11].

2.2 Preparation of Chitin Solution (substrate)

Chitin solutions were ready with some modifications as described below. Used the following procedure for the preparation of Colloidal Chitin. 10 grams' powder of chitins weregradually in 400 ml of concentrated of hydrochloric acid and kept on magnetic stirring at 4 ° C, Then the blend of chitin is kept in a water bath at 37 ° C until the consistency is reduced,[3]mix this solution with 4.0 ml of Sterilized distilled waterincluded then was placed for 4 °C for one night. A condensed white deposit is after that used the centrifugation method at 10 minutes. The sediment was then washed repeatedly in wet water to reach pH 5.5. The supernatant is lost and then the colloid chitin is stored in the refrigerator for future use [24].

2.3 Select of sample

Soil samples were collected from Agricultural soils and were separated by plate weakening strategy. Single confines were filtered by streaking to potato dextrose agar or Sabouraud dextrose agar [18].

2.4 Isolation of fungi samples

Regulated strains were utilized to isolate fungi. On the area of potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA), 0.1 ml sample of homogeneous strains was developed by L-rod [4].Then dishes were kept for three or five days, after incubation, you will see the morphological fungi which they were added to PDA agar dishes [25].

2.5 Screening of chitinase activity

On chitin-agar media, fungal strains isolated for activity of the chitinase. According of 1000 ml of the chitin –agar media which containing, (NH_4) 2 SO_4 (1g), K $_2HPO_4$ (1g), KCl (0.5g), NaCl (5g), $MgSO_4$ (0.5g), $FeSO_4$ (0.01g), agar agar powder 20grams chitin solution (5g). Then a part of the insolated fungus cultural added and after addition of fungus strains these solutions were kept for 6 to 7 days, at room temperature at the chitin agar plates. The activity of chitinases were detected with circle zone of releases on colonies of fungal, by adding red colored Congo solution[10].

2.6 Identification of Aspergillus niger, producing chitinase

Chitinase enzyme which product from fungus encountered distinguishing proof dependent morphological qualities and cultural [5]. Development shading, surface and colors were visibly watched and the construction of fungi was determined by consume of microscopy lactophenol cotton blue [14].

2.7 Production of chitinase

In 250 ml conical cup Production of chitinase which have 50 ml of medium, chitin (5 grams), (g / L) (NH_4) 2 SO_4 (1 gram), K $_2HPO_4$ 1 gram, $MgSO_4$ 0.5 gram, KCl (0.5g), NaCl (5g) and $FeSO_4$ (0.01g). The flask was suspended by (1 ml) suspension of fungus and then kept for 7 days at 30 ° C below of shaky conditions to 160 rpm[10].

2.8 Purification of Chitinase Enzyme

The unrefined proteins (enzymes) reactions were hastened with 75% w/v ammonium sulfate. The solution was incubated at 4 ° C on one night. The pellets of tubes were reused with centrifugation of protocol and after that broke up into 50 mM phosphate buffer support that pH were 7.0. The 24-hour suspension was analyzed versus distilled water, and arrangements that were acquired after hydrolysis with dialysis membrane and absorbed the results [10].

2.9 Chitinase activity

Activity and movement of Chitinase was controlled by evaluating the measure of free development shaped by substrate hydrolysis like chitin solution. This response includes of 1 ml of substrate or chitin solution(within 0.**02MphosphatebufferpH** 7) **also** (0.5 ml) soluble enzyme. The mixture was incubated for 1 hour at 30 ° C. Thisyield was estimated in a milliliter of filter utilizing Dinitrosalicylic corrosive (DNS) strategy utilizing N-acetylglucosamine like standard protocol. Then, using a UV-Vis spectrophotometer with a wavelength of 540 nm[11].

3. Results

Fungi are eukaryotic organisms and heterotrophic organisms, except of yeasts they are aerobic and abundant in surface soils so important on decomposition of organic matter so soils contain millions of fungi per gram is diluted using a dilution series which is made by given amount of soil in a solution.

To isolate secondary metabolites (enzymes, proteins, antibiotics etc…) producing fungi from soil samples.

Serial dilution method is one of the oldest and usable method which is use for the isolation of fungi as well as for the isolation of viable bacterial colony.

In this method we collected our desire samples (soil, slag, water, milk, food) and make its dilution in the test tubes (Master Test Tube).

We inoculated the sample from the diluted test tubes in the prepared medium plates by using pure plate method or spread method and then incubated the inoculated plates in the incubator at 28^0 C (for Fungus growth) for 48 to 72 hours or one week.

After the specific growth duration of the microbes (fungi) we absorb the cultured plates, the growth will appear on each plates.

Next we performed sub culturing method for the isolation of pure culture. After isolation of pure culture, we perform biochemical test for the conformation and identification of fungi species.

3.1 Microbial Tests

3.1.1-Counting of colonies

Pouring the sample should be done in a variety of ways, as the sample is dense in nature, and there are more microorganisms in the sample that can be difficult to count. Therefore, the serial dilution must be done to count colonies.

To dilute the serial, 9ml (distilled water), 1 ml (sample) should be placed in a test tube.

3.1.1.1 Colony Forming Unit

This is a unit for measuring the number of acceptable cells in a particular sample, which is the number of cells that are active in the division of the cell.

The serial dilution of 30 to 300 colonies should be easily counted. Plates counting with more than 300 colonies are counting. Plates with fewer than 30 colonies are less for counting The basic calculation of colony framing unit are:

Cfu/ml=number of colonies x dilution factor/volume of culture plate.

The dilution factor used for sample is 10^{-1}, 10^{-3}, $10^{-4,}$ and 10^{-8} dilution.

- At 10^{-1} dilution colonies formed which are 8 in number

Cfu/ml =8 x 10^{-1}/1000 = 0.08 colonies/ml

- At 10^{-3} dilution there is formation of colonies which is 4 in number

Cfu/ml = 4 x 10^{-3}/1000 = 4 colonies/ml

- At 10^{-4} dilution there is formation of colonies which is 2 in number

Cfu/ml = 2 x 10^{-4}/1000 = 20 colonies/ml

- At 10^{-8} dilutions number of colonies increases which is 6 in counting

Cfu/ml = 6 x10^{-8}/1000 =60000 colonies /ml

3.1.2-Isolation of Fungal Colonies

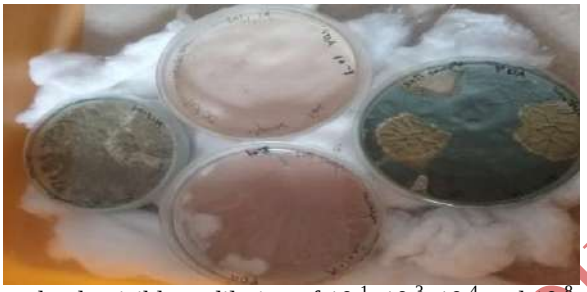
Thedecant plate technique is utilized for the purpose of isolation. The Pour plate method is used for anaerobic microorganisms. A laminate for separation is used to expand aerobic microorganisms. Fungus colonies are used on

various media plates used to count and also isolate certain bacteria, including various media:

3.1.2.1PDA (Potato Dextrose Agar)

PDA is used for Separation, counting and culture of yeasts and molds are used from the sample, and therecognizable proof of organisms and yeasts, alongside cell morphology or micro-culture techniques into the slide is used. Agar is a solidifying agent in the medium for counting the fungus and counting yeast and mold [2].

It's a choice and its different agar media. Basically it is used to count the growth of microorganisms that are impossible in nature. Isolation and clearing of cultures is done through PDA. Pouring the sample can be done with different dilutions (**Figure 1**). The number of colonies increases with increasing dilution concentration; The Colonies are



clearly visible at dilution of 10^{-1}, 10^{-3}, $10^{-4,}$ and 10^{-8}.

Figure 1. Pouring of samples on PDA at different dilutions.

3.1.3-Sub- culturing and Purification

3.1.3.1 Nutrient Agar

The sub- cultivation of cultivated colonies from the sample can be isolated again for purification purposes. Streaking method can also be applied to agar nutrient.

3.1.3.2 Sabouraud Dextrose Agar (SDA)

Sabouraud Dextrose Agar (SDA) is utilized for the segregation, development, and upkeep of non-pathogenic and pathogenic types of fungus [19]. Sabouraud dextrose agar dishes can be vaccinated by streaking method, similarly like with standard media of bacteriological, or with presenting the medium to surrounding air, kept and brooded at temperature room (22 to 25°C). Recognizable proof of micro-living-organism is performed by watching different parts of settlement morphology, trademark minute structures, rate of development, media which underpins the living being's development.

3.1.4 Screening of Chitinase Activity

3.1.4.1 Colloidal chitin

Colloidal Chitin is used Chitinolytic micro-organisms that produce chitinase[16]. Chitins aren't freely water; chitins are frequently like chemically formulated as a chitin colloidal, with small particle sizes that can easily be scattered (**Figure 2**). Homogeneous in agar media is consistent, and consistent[15].

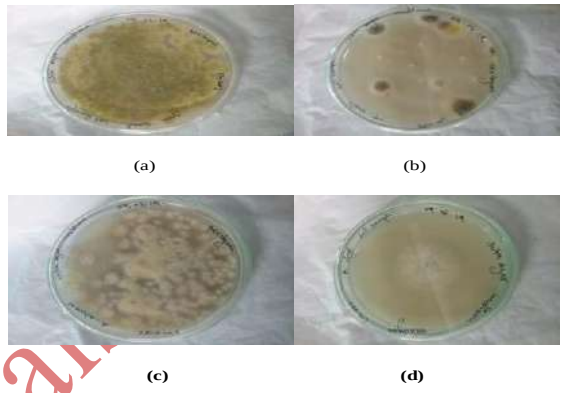


Figure 2. Growth of different fungus on chitin agar media (a) Green fungi, (b) Tricodermata. reesie (c) Aspergillus ochrceus and (d) Aspergillus niger.

The nearness of clear radiance around the settlements shows activity and movement of the fungi. In this study, strain of the fungi: such us Aspergillus niger, Aspergillus ochraceus, Tichodermata. ressie Aspergillus. niger demonstrated positive outcome for chitinase creation so between three, one greatest strain such us Aspergillus niger was selected for further study. The most favorable temperature and pH are $30c^0$ temperature and 4 and 5 pH for Aspergillus niger on chitin agar media for screening of chitinase activity.

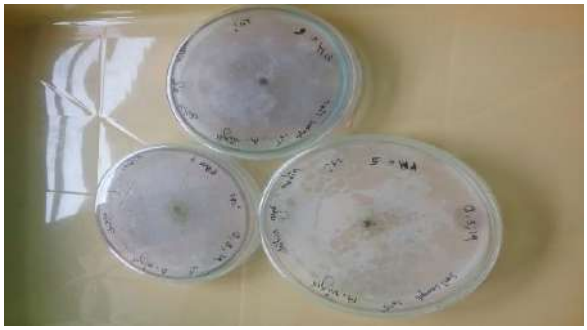


Figure 3. Growth of Aspergillus niger on chitin agar medium at pH 4,5 and 6 on $28c^0$ incubation

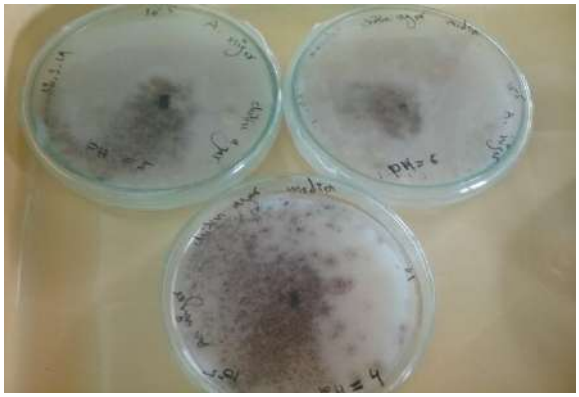


Figure 4. Growth of Aspergillus. niger on chitin agar medium at pH 4, 5 and 6 on 30c^0 incubation

3.1.5Identification of Aspergillus niger, producing chitinase

Aspergillus niger strain was recognized by considering social and morphological attributes, by using lacto phenol cotton blue which is highly broadly utilized strategy for recoloring and watching growths and has three segments (Table 1)[13].

1. Which Phenol kills living beings.

2. Lactic corrosive it jams fungus structures

3. Cotton blue which it recolors the chitin in the contagious cell dividers.

Table 1. Morphological characters of A. niger

No	Characteristic	A. niger
1	Colony color	Black
2	Colony appearance	Spread spores
3	Conidial head	Present
4	Colony texture	Smooth
5	Surface of colony	Smooth colony
6	Vesicle shape	Ovaid
7	Rhizoids	-
8	Sporangium	-
9	Conidia	Shape

3.1.6-Production of chitinase

After 7 days of incubation, chitinase was produced using the best strain, the culture containing the centrifuge production medium and the supernatant containing Enzyme like crude enzyme.

3.2 Biochemical Test

3.2.1-Purification of chitinase

At the point when the samples were dialyzed and focused. This demonstrated the degree of cleansing for each situation, then use for chitinase activity purpose.

3.2.2-Estimation of chitinase activity

The calorimetrically assay chemical movement was determined and the qualities determined with the presence of the standard N-acetylglucosamine values were calculated, so the complete protein value was determined on the basis of scale values.

3.2.3- Standard Curve

The standard curve for N- acetyl D- glucosamine was prepared with help of DNS reagent. Standard concentrations were treated with DNS reagent and the absorbance was recorded, with concentration of 200 micrograms per ml.

Figure 5. N- acetyl D- glucosamine Standard by DNS method

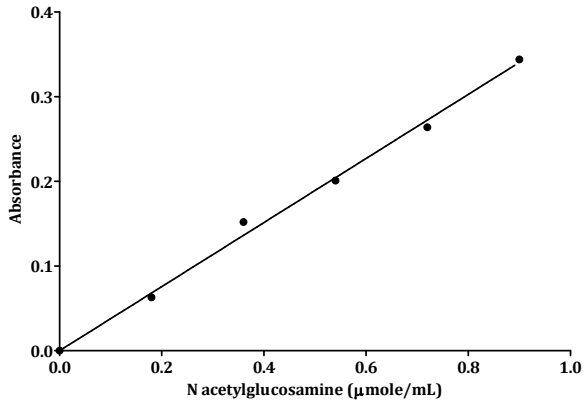


Figure 6. N- acetyl D- glucosamine Standard Graph by DNS method

The activity of enzymes was evaluated calorimetrically and the qualities determined utilizing standard estimation of standard N - acetylglucosamine. The activity of enzyme and their concentration created by example of Aspergillus niger(**Table 2**).

Table 2. Activity of Enzyme at various substrate Concentration

Sample	Substrate concentration (mg/ml)	Net Absorbance	Product formed per 0.5 ml extract (µmoles/ 60 min.)	Enzyme activity Per ml of crude extract (µmoles/min)
Aspergillus niger	10	0.113	0.2986386	0.0099
	10	0.162	0.4281367	0.0142
	5	0.160	0.422851	0.0140
	2	0.20	0.5285638	0.0176
	2	0.140	0.3699947	0.0123
	1	0.063	0.1664976	0.0055

Conclusion

In this study, chitin is a standout amongst the most bounteous complex carbohydrate to ground. A great deal of chitineous material is making in the oyster of prawn, crayfish or marine animal like lobsters.

Chitinases are generally expressed into microscopic life form. The quantity of microorganisms like fungus having a place with the sort Aspergillus, for example, Aspergillus. niger, has been confirmed as a Chitinase manufacturer. In this species, Aspergillus.niger showed that the greater enzyme, which was treated on the colloidal plates and raw chitin, showed a strong and high kinolytic acting. In this investigation, chitin colloid arranged from chitin shell powder was utilized as a substrate and a higher measure of chitinase compound was gotten. Purging of the chitinase protein delivered via the Aspergillus type was performed via dialysis layer. These pure chitinases were used to determine the chitinase assay for the evaluation of chitins' activity and protein concentration.

The fungal strain like, Aspergillus niger contain be distinguished as great chitinase producer confined from soil. They were affirmed that, the filtered protein had the capacity to corrupt chitin substances. To futurity intrigue will derived with increase the generation of chitinase by utilizing different agro deposits in strong state maturation

A fungal strain such as Aspergillus niger has been identified as well-produced chitinase from soil. They were affirmed that the unadulterated protein had the capacity to destroy the kitein material. In the future, there will be an enthusiasm for expanding the generation of protein by utilizing different strong buildups in strong aging.

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