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Optimization of chitinase synthesis or chito-oligosaccharide production using antibiotic sensitive *Cellulosimicrobium cellulans* isolated from mushroom compost

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Abstract
Chito-oligosaccharides are having increasing applications in fields like food, cosmeceutical, waste management, agriculture and medicine. Chito-oligosaccharides play important role in plant defense. There is a great demand for high production of chito-oligosaccharide with superior properties. Physical and chemical methods of chito-oligosaccharide production demands high energy inputs or they are not environment friendly. For this better option is enzymatic production. In the present work, attempts are made for enzymatic production of chito-oligosaccharide using chitinase producing culture. For achieving this, screening, isolation of chitinase producer (chito-oligosaccharide yielding) organism, optimization of fermentation conditions for chitinase production and finding optimum conditions for chitinase activity was done. Chitinase producing Cellulosimicrobium cellulans strain IARI-ABL-30 was isolated from mushroom compost. Organism was sensitive to commonly used antibiotics hence can be safe organism for production of chito-oligosaccharides from chitin. Meat extract, 45oC, pH 7 and incubation period of 4 days are optimum conditions for chitinase mediated chito-oligosaccharide production from biological chitin by Cellulosimicrobium cellulans strain IARI-ABL-30.

Keywords: chitinase, *Cellulosimicrobium cellulans*, chito-oligosaccharides, N-acetyl D-glucosamine

Introduction
High molecular weight nontoxic polymeric compounds - chitin and chitosan - have limited solubility at pH near neutrality. As a result, their applications in food, pharma and agriculture sectors are restricted. This limitation of solubility is not observed in oligosaccharides derived from hydrolysis of chitin and chitosan [1]. Oligosaccharides derived from chitin and chitosan are known as Chito-oligosaccharides.

Chito-oligosaccharides find wide applications in the pharmaceutical industry. Chito-oligosaccharides show antioxidant, anti-inflammatory and anti- HIV activity [2]. Chito-oligosaccharides exhibit defense response in plants against microbial infections. As a result they are helpful in improving quality and quantity of crops [3]. Chito-oligosaccharide and starch complex showed anti-obesity effect in experimental rats fed a high fat diet [4]. During healing of wound, for skin tissue regeneration, a scaffold containing fish collagen-alginate and chito-oligosaccharide gave superior results [5]. Chito-oligosaccharides also act as prebiotic by improving gut microbial flora and decreasing the possibility of pathogen survival in gut [6]. In pigs, supplementation of chito-oligosaccharide in diet increased count of probiotic bacteria - *Bifidobacteria* and *Lactobacilli* while reducing the population of pathogen *S. aureus* [7] and it also helped in nutrient absorption and digestion [8]. Chito-oligosaccharides are also important in plant protection. Plants have developed a mechanism that can identify many molecules present in the pathogens [9]. Chito-oligosaccharides can act as a plant vaccine by providing protection against diseases. It also stimulates plant growth and prevents injuries caused by chilling [10].

Chito-oligosaccharides are synthesized by hydrolytic cleavage of chitin or chitosan, recovered from shells of crustaceans like shrimp, prawns, crabs, etc. [11]. Chito-oligosaccharides are synthesized by various methods including physical, chemical and enzymatic. Physical methods include hydrothermal treatment in presence of tartaric acid, gamma radiations, microwave irradiation and high intensity ultrasound waves [12, 13, 14, 15]. Chemical method of chito-oligosaccharide synthesis includes use of concentrated HCl, concentrated H_2SO_4, H_2O_2, oxidative degradation in presence of $NaNO_2$ and ozone [16, 17, 18, 19, 20, 21]. These chemical extraction methods are not environment friendly since use of corrosive chemicals generates harmful waste water. In recent years, enzymatic production of chito-oligosaccharides has evolved as it is performed under very mild conditions [22]. For chito-oligosaccharide formation from chitin, chitinolytic enzymes are used. Chitinolytic enzymes belong to glycoside hydrolases and hydrolyze the glycosidic bond between N-Acetyl D-Glucosamine sugar molecules (NAG-NAG). Chitinolytic enzymes - chitinases occur in families, glycoside hydrolases 18 (GH18) and glycoside hydrolases 19 (GH19) [23, 24].

A wide range of organisms, ranging from bacteria, molds, insects, plants to animals including humans, can synthesize chitinases. In each of these, chitin serves a different purpose [25]. Bacteria synthesize chitin mainly for its utilization as a carbon and nitrogen source and as virulence factor [26, 27].

Materials and Methods
Materials
All the chemicals used for experiments were of analytical grade. For isolation of chitinase producing organism from

various chitin rich sources, samples like rhizosphere soil, mushroom compost and soil of shrimp waste disposal area (Nashik, India), marine water and marine sediment (Sindhudurg, India) were collected.

Methods

Isolation and screening of chitinase producing organisms

Enrichment of chitinase producers was done in medium containing colloidal chitin as the sole carbon source therefore; it selectively allows growth of chitinase producing organisms [28]. In this medium, 1% of each sample were added and incubated on shaker at room temperature (c. a. 25ºC) at 100 rpm for 48 hours. After enrichment, 0. 1 ml of enriched broth was spread on colloidal chitin agar plates. Colonies showing zone of clearance against turbid background were selected as chitinase positive cultures and maintained on nutrient agar.

Selection of potent chitinase producer by chitinase assay

All the selected chitinase positive cultures were inoculated in colloidal chitin broth in Erlenmeyer flasks on rotary shaker at 100 rpm. After 4 days of incubation at room temperature (c. a. 25ºC), broth was centrifuged at 10000 rpm for 10 minutes at 4ºC. For chitinase assay, amount of reducing sugar released from colloidal chitin by chitinase was measured by 3, 5 dinitrosalicylic acid reagent.

Mixture containing 1 ml each of centrifuged cell free broth, 0. 1 M phosphate buffer of pH 7 and colloidal chitin was incubated on shaker at 100 rpm for 1 hour at room temperature (c. a. 25ºC). Detection of potent chitinase producer was done by measuring amount of reducing sugar released from chitin by 3, 5 dinitrosalicylic acid assay [29]. After 1 hour chitinase activity was stopped by addition of 3, 5 dinitrosalicylic acid reagent. On centrifugation of this mixture at 10000 rpm for 10 minutes, supernatant was boiled for 10 minutes and absorbance was measured by UV-Visible spectrophotometer at 540 nm. Amount of reducing sugar released was measured using standard graph of N-acetylglucosamine. The amount of enzyme required to release 1µM of N-acetylglucoseamine (NAG) in one minute is defined as one unit of chitinase activity under above mentioned conditions.

Identification of chitinase producing organism

Culture showing maximum chitinase production was subjected to identification by 16S rRNA sequence at Chromous Biotech Private limited, Bengaluru, India. Genomic DNA was isolated from the bacterium. The ~1. 3 kb/1. 5kb, 16S rRNA fragment was amplified using high–fidelity PCR polymerase. The PCR product was sequenced bi-directionally. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors.

The PCR product size was ~1. 5 kb.
Primers used for PCR for amplification were
16S Forward Primer: 5'-AGHGTBTGHTCMTGNCTCAS-3'
16S Reverse Primer: 5'-TRCGGYTMCCTTGTWHCGAC TH – 3'

PCR was performed using ABI 3500 Genetic Analyzer with a cycle of 96ºC for 5 min for Initial denaturation, 96ºC for 30 seconds for denaturation, 50°C for 30 seconds for hybridization and 60ºC for 1. 30 min for elongation.

Antibiotic sensitivity of Cellulosimicrobium cellulans

Potent chitinase producer organism (M 3) was checked for antibiotic sensitivity at Bactochem Laboratory, Nashik, India. Bacterial suspension having 0. 5 McFarland turbidity standards was diluted in 0. 45% saline up to 1. $5x10^7$ CFU/ml. In the VITEK 2, cards were filled automatically, sealed and loaded in the instrument and incubated for reading. Depending on bacterial culture antibiotics were selected for antibiotic sensitivity testing [30]. Antibiotic sensitivity of selected organism was done by finding Minimum Inhibitory Concentration (MIC).

Study of nature of chitinase

Checking induced / constitutive nature of chitinase

To find whether the enzyme chitinase in *Cellulosimicrobium cellulans* strain IARI-ABL-30 is constitutive or induced, culture was grown in colloidal chitin medium which does not contain chitin. Regular colloidal chitin medium was used as a control. After incubation, cell-free broths were taken for chitinase assay by 3, 5 dinitrosalicylic acid method.

Checking cell-free (dissolved) / cell bound nature of chitinase

To check whether chitinase in *Cellulosimicrobium cellulans* strain IARI-ABL-30 after synthesis remains free in the medium (dissolved) or bound to the outside of the cell, culture was inoculated in colloidal chitin broth. After incubation cells were separated and added in saline and then vortexed for 10 minutes to loosen cell bound chitinase and chitinase activity of cell-free saline was checked by 3, 5 dinitrosalicylic acid method.

Optimization of chitinase production

Influence of temperature

For finding optimum temperature required for chitinase production, 6 flasks containing colloidal chitin were inoculated with 24 hours old culture of *Cellulosimicrobium cellulans* and incubated at different temperatures (30ºC, 35ºC, 40ºC, 45ºC, 50ºC and 55ºC) in shaker incubator for 48 hours. After incubation, centrifuged cell free broth (10, 000 rpm for 10 minutes at 4ºC) from each flask was used for chitinase assay.

Influence of pH

For deciding optimum pH of the medium for chitinase production, colloidal chitin broth with different pH (4, 5, 6, 7, 8, 9 and 10) were inoculated with 24 hours old culture of *Cellulosimicrobium cellulans* and incubated at 45ºC for 48 hours. After incubation, centrifuged cell free broth (10, 000 rpm for 10 minutes at 4ºC) from each flask was used for chitinase assay.

Influence of incubation period

To find out the incubation period required for optimum production of chitinase, in 100 ml colloidal chitin broth 24 hours old culture of *Cellulosimicrobium cellulans* was inoculated and incubated in shaker incubator at 45ºC. After every 24 hours samples were withdrawn under aseptic condition. Centrifuged cell free broth (10, 000 rpm for 10 minutes at 4ºC) was used for chitinase assay.

Influence of nitrogen source

To optimize chitinase production, different nitrogen sources were incorporated in colloidal chitin broth. In original

colloidal chitin broth, NH_4Cl is the main nitrogen source. Different media containing 3 different organic and 3 inorganic compounds were added in separate medium instead of NH_4Cl. Media were inoculated with 24 hours old culture of *Cellulosimicrobium cellulans* and incubated at 45ºC for 48 hours. After incubation, centrifuged cell free broth (10, 000 rpm for 10 minutes at 4ºC) from each flask was used for chitinase assay.

Results and discussion

Isolation and screening of chitinase producing organisms

For chitinase mediated synthesis of chito-oligosaccharide, a systematic screening of chitinase producing organism was done.

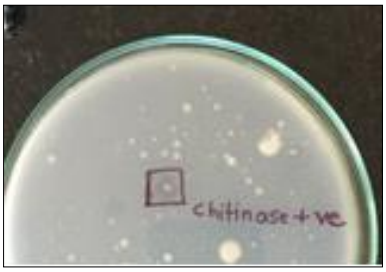

Fig 1: Zone of clearance around chitinase producer colony on colloidal chitin agar.

After 48 hours incubation, enriched samples from various chitin containing sources like rhizosphere soil, mushroom Compost, shrimp waste disposal area, marine water and marine sediment were plated on colloidal chitin agar. As shown in Figure 1**,** colonies with zone of clearance on colloidal chitin agar plates showing difference in colony morphology were selected as chitinase positive organisms. Total 22 chitinase producing bacterial and fungal isolates were isolated as shown in Table 1. All the isolates were maintained on nutrient agar in refrigerator at 4ºC with routine sub-culturing. Majority of soil bacteria displayed potent chitinolytic activity and hence play a vital role in recycling of chitin in soil [31]. Rashad *et al.,* observed that majority of isolates from marine environment showed chitinase activity [32]. In the present study, from soil and marine environment, many chitinolytic organisms were isolated.

Selection of potent chitinase producer using 3, 5 dinitrosalicylic acid assay

All 22 isolates were checked for quantitative chitinase assay by 3, 5 dinitrosalicylic acid method using colloidal chitin as a substrate. Many researchers screened chitinase producers using this method [29, 33, 34, 35].

Chitinase positive cultures were inoculated in colloidal chitin broth in Erlenmeyer flask. After 4 days of incubation, cell free broths were assayed for amount of reducing sugar released from colloidal chitin. Among all 22 isolates, isolate 'M 3' showed significantly higher (10. 86 U/ml) enzyme activity than other cultures and hence it is selected for further study.

Table 1: Chitinase enzyme activity of isolates by DNSA method

Sr. No.	Site	Isolate No	Chitinase activity U/ml
1	Rhizosphere soil	RS 1	9. 11
2		RS 2	2. 34
3		RS 3	5. 45
4		RSF 4	4. 65
5		RSF 5	3. 42
6	Mushroom compost	M 1	4. 60
7		M 2	2. 97
8		M 3	10. 86
9		M 4	7. 46
10		MF 5	10. 02
11		MF 6	8. 09
12	Marine water	MW 1	6. 43
13		MW 2	5. 48
14		MW 3	2. 98
15		MW 4	5. 54
16	Marine sediment	MS 1	4. 58
17		MS 2	3. 87
18		MS 3	4. 69
19		MS 4	2. 96
20		MS 5	4. 06
21		MS 6	3. 40
22		MS 7	8. 87

Identification of chitinase producing organism

Selected potent chitinase producer isolate 'M 3' was Gram positive, motile, short rod shaped bacteria. Identification was done by 16S rRNA sequencing.

16S rRNA sequencing

Based on sequencing studies the potent chitinase producer isolate 'M 3' was confirmed as *Cellulosimicrobium cellulans* strain IARI-ABL-30. Aligned Sequence Data of *Cellulosimicrobium cellulans* strain IARI-ABL-30 was used for identification.

TTCGTAGATCGTGGCATCAGTGTACAACGATGAAT
CCCACTTGCTGGGTGGATTAGTGGCGAACGGGTGA
TTAACACGTGAGTAACCTGCCCTTGACTTCGGGAT
AACTCCGGGAAACCGGGGCTAATACCGGATATGAG
CTACCTTCGCATGGGGGTGGTTGGAAAGTTTTTCG
GTCAGGGATGGGCTCGCGGCCTATCAGCTTGTTGG
TGGGGTGATGGCCTACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTG

AGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGCAAGCCTGATGCA
GCGACGCCGCGTGAGGGATGAAGGCCTTCGGGTTG
TAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGAC
GGTACCTGCAGAAGAAGCGCCGGCTAACTATGTGC
CAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTG
TCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGG
TCTGTCGCGTCTGGTGTGAAAAACTCGAGGCTCAA
CCTCGAGCTTGCATCGGGTACGGGCAGACTAGAGT
GCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGT
GGAATGCGCAGATATCAGGAGGAACACCGATGGC
GAAGGCAGGTCTCTGGGCCGCACTGACGCTGACGA
GCGAAAGCATGGGGAGCGAACAGGATTAAATACC
CTGGTAG

Phylogenetic Tree- of M3 is shown in Fig. 2 and isolate 'M 3' is represented as 3T

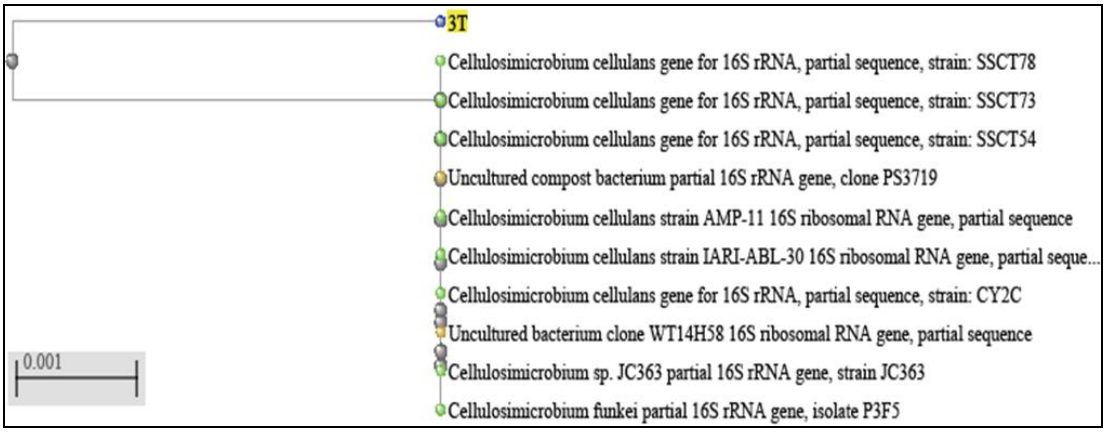

Fig 2: Phylogenetic tree- of M3 is shown in

Cellulosimicrobium cellulans is generally regarded as safe organism, as infection by this organism is very rare and may cause infection only in immunocompromised individuals [36]. *Cellulosimicrobium* spp. are known for production of various degradative enzymes like protease, cellulase, β-glucosidase, glycoside hydrolase along with chitinase [37].

Antibiotic sensitivity of Cellulosimicrobium cellulans strain IARI-ABL-30

The application of chitinase producer *Cellulosimicrobium cellulans* strain IARI-ABL-30 for chito-oligosaccharide production may cause release of culture into the environment. The antibiotic sensitivity of this strain was carried out to ensure its control in case it caused any infection.

Table 2: Antibiotic sensitivity of *Cellulosimicrobium cellulans* strain IARI-ABL-30

Antibiotic	Sensitivity	Antibiotic	Sensitivity
Amikacin	S	Gentamicin	S
Amoxyclav	S	Imipenem	S
Ampicillin	S	Levofloxacin	S
Cefaparazone	S	Linezolid	S
Cefotaxime	S	Meropenem	S
Cefoxitin	R	Netillin	S
Cefpodoxime	R	Nitrofurantoin	S
Ceftazidime	S	Ofloxacin	S
Cetriaxone	S	Oxacillin	R
Cetriaxone/Sulbactam	S	Penicillin	R
Cefuroxime	R	Piperacillin	S
Ciprofloxacin	S	Rifampicin	S
Clindamycin	S	Tetracyclin	S
Co- Trimoxazole	S	Tigecycline	S
Doxycycline hydrochloride	S	Vancomycine	S
Erythromycin	S		

"R"- Resistant, "S"-Sensitive

Commonly recommended 31 antibiotics were tested against the strain. The strain showed sensitivity towards 26 antibiotics as shown in Table 2. From this result it could be inferred that this strain can be safely used for production of chito-oligosaccharides from chitin. If by chance it produces infection, it can be cured immediately using commonly used antibiotics and it will not pose a problem of drug resistance. Therefore, chitinase producer *Cellulosimicrobium cellulans* was considered as an efficient and safe organism for production of chito-oligosaccharides from chitin.

Study of nature of chitinase

Checking induced / constitutive nature of chitinase

To check induced or constitutive nature of chitinase enzyme of *Cellulosimicrobium cellulans* strain IARI-ABL-30, culture was grown in colloidal chitin broth with and without colloidal chitin. After centrifugation, cell free broth was added in a well on colloidal chitin agar for observation of zone of clearance. Cell free broth of *Cellulosimicrobium cellulans* strain IARI-ABL-30 grown in a medium without colloidal chitin showed no zone of clearance around the well. It suggests that chitinase production in *Cellulosimicrobium cellulans* strain IARI-ABL-30 is induced and not constitutive. Similar reports were observed for chitinase of *Aeromonas* strain. It was also secreted inductively in presence of abundant chitin in habitat and gene required for chitinase production -*chi*SL in *Bacillus pumulus* is induced in presence of chitin and repressed in presence of glucose [38, 39].

Checking cell-free (dissolved) / cell bound nature of chitinase

High molecular weight substrate like chitin need to be converted into molecular fractions smaller than 600 daltons, hence chitinases are extracellular. Extracellular enzymes can be freely present in the broth or may be cell-bound [40]. For this cells and broth were separated. After vortexing cells in saline, no chitinase activity was observed in saline which suggests absence of cell-bound chitinases in *Cellulosimicrobium cellulans* strain IARI-ABL. Chitinase activity was observed only in cell-free broth which suggests that chitinase of *C. cellulans* strain IARI-ABL is freely

present in the broth. Majority of bacterial chitinases are secreted freely and only *Haloferax mediterranei* chitinase was cell-bound [41].

Optimization of chitinase production using biologically recovered chitin as substrate

Influence of temperature

Incubation temperature shows remarkable difference in the production of chitinase enzyme by *Cellulosimicrobium cellulans* as shown in Fig. 3 Maximum yield of 21. 51 Units/ml was obtained at 45ºC. When temperature of incubation increased from 30 to 45ºC, production of chitinase increased gradually, but after 45ºC, chitinase production started declining. Optimal chitinase production by *Cellulosimicrobium cellulans* was also seen at high temperature (45ºC), in the present study.

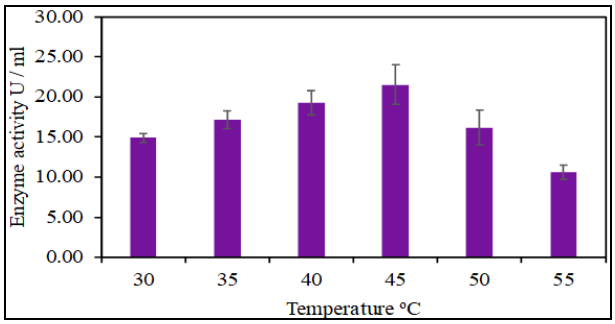

Fig 3: Influence of temperature on chitinase production by *Cellulosimicrobium cellulans*

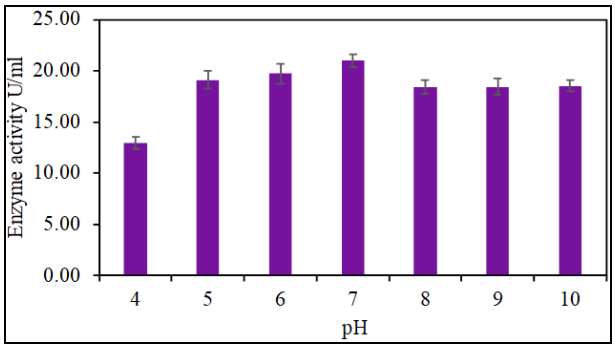

Fig 4: Influence of pH of medium on chitinase production by *Cellulosimicrobium cellulans*

Many bacteria show maximum chitinase production at 40ºC [42]. Ueda and Kurosava [43] isolated *Paenibacillus thermoaerophilus* from compost which is capable of producing chitinase at 50-55ºC (thermophilic).

Influence of pH

The initial pH of medium plays a crucial role in chitinase production. *Cellulosimicrobium cellulans* produces maximum chitinase activity (20. 99 U/ml) at neutral pH as shown in Fig. 4 Chitinase production was affected to a very small extent when pH of the medium was acidic (pH 5) (from 20. 99 to 19. 33 U/ml). As compared to alkaline pH, under acidic conditions, chitinase production is greater.

Similar production pattern was observed for *Bacillus licheniformis* SSCL10 and *Enterobacter* sp. which showed maximum production near neutral pH [44, 34]. *Chitinolyticbacter meiyuanensis* SYBC-H1 showed maximum production at pH at 7. 5 [45] and *Alkaligens xylosoxydans* showed pH optima at 8 [46].

Influence of nitrogen source for chitinase production

In the present study, the medium referred by Kuddus & Ahmad [28] was used for the initial chitinase production experiments. It contains ammonium chloride as the source of nitrogen. For selection of best nitrogen source for chitinase production, different organic and inorganic Nitrogen sources were checked at 0. 2 and 0. 1% concentration respectively. Meat extract, soyabean meal and casein digest (3 organic) and ammonium chloride, potassium nitrate, ammonium nitrate and ammonium di hydrogen phosphate (4 inorganic) nitrogen sources were checked for chitinase production by *Cellulosimicrobium cellulans* strain IARI-ABL-30. As shown in Fig. 5, in medium containing meat extract, a maximum yield of 18. 67 U/ml was observed. Hence in further experiments, instead of ammonium chloride, meat extract was used. The results indicate that organic nitrogen is more effective than inorganic nitrogen sources for production of chitinase using *Cellulosimicrobium cellulans*. Studies have indicated the effectiveness of organic nitrogen over inorganic one. Organic nitrogen, such as malt extract, readily provides assimilable nitrogen along with growth factors augmenting enzyme production [47]. Dhar and Kaur also showed that chitinase production in *Metarhizium anisopliae* is enhanced in presence of organic nitrogen such as yeast extract. Similar results have been reported by Sandhya *et al.* and Jha *et al.* [48, 49, 50].

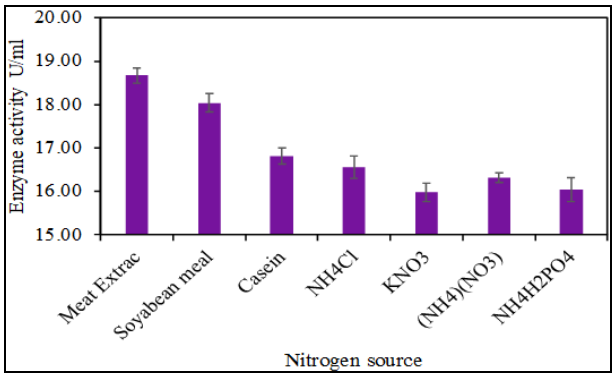

Fig 5: Influence of nitrogen source on chitinase production by *Cellulosimicrobium cellulans*

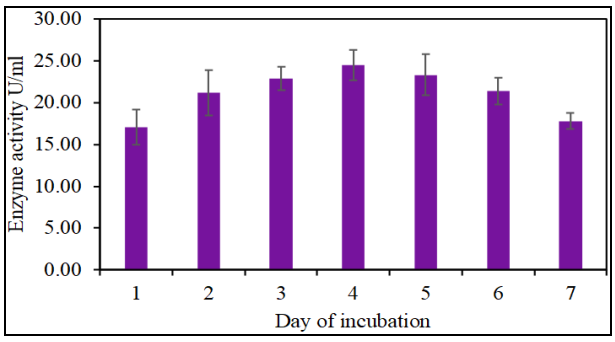

Fig 6: Influence of incubation period on chitinase production by *Cellulosimicrobium cellulans*.

Incubation period

Chitinase production was done using modified medium of Kuddus & Ahmad (containing meat extract as nitrogen source instead of ammonium chloride), pH 7 and optimized incubation temperature of 45ºC [28]. After 4 days of incubation (96 hours) as shown in Fig. 6, maximum (24. 51 U/ml) of chitinase was produced by *Cellulosimicrobium*

cellulans. Alhasawi and Appanna [51] reported 6 days for optimum chitinase production by *Pseudomonas fluorescens* which is longer than reported in the present study.

Conclusion

From the above study it can be concluded that Chitinase producing bacterium *Cellulosimicrobium cellulans* strain IARI-ABL-30 was isolated from mushroom compost. *Cellulosimicrobium cellulans* was considered as an efficient and safe chitinase producing organism due to its sensitivity to commonly used antibiotics. Chitinase production in *Cellulosimicrobium cellulans* strain IARI-ABL-30 is induced and not constitutive. chitinase of *C. cellulans* strain IARI-ABL is extracellular and hence freely present in the cell broth. Optimum production of chitinase by this organism is possible by using Meat extract as Nitrogen source, incubation temperature 45ºC, pH 7. Maximum production takes place after incubation period of 4 days. This synthesized chitinase mediated chito-oligosaccharide production from biological chitin by *Cellulosimicrobium cellulans* strain IARI-ABL-30.

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