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Molecular characterization of yeast endophyte isolated from leaves of *Capsicum assamicum* of Assam and screening for its cellulase and lipase enzymes

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Abstract
Yeast endophytes are considered rare and occupy a small population of almost 6% in the entire endophytic community. They outshine bacterial and fungal endophytes in quite few aspects. In this study, yeast endophytes were targeted for isolation from young and healthy leaves of *Capsicum assamicum* from Guwahati region, Assam. It was further characterized using 18s rRNA ITS sequencing and phylogenetic analysis. Cellulase and lipase secreting potential of the isolated yeast endophyte was done on PDA plates supplemented with carboxymethyl cellulose (CMC) and Tween 20 respectively. Results revealed the presence of a pink pigmented yeast species and molecular characterization identified it as *Cystobasidium calyptogenae*. Also it exhibited good potential ability of lipase and cellulase secretion by clear zone formation around the colonies. However, this study further requires enzyme quantification assays and also this yeast endophyte could be used in biodiesel production and industrial bioconversion of cellulose.

Keywords: yeast endophyte, *c.assamicum*, *c.calyptogenae*, cellulase, lipase, its sequencing

Introduction
Endophytic study remains a highly fascinating and demanding arena of research in current scenario. Bacterial and fungal endophytic research has been carried and explored in terms of their secondary metabolites but yeast endophytic biology still remains very poorly understood and unclear. This notable rarity may be attributed to the complex and cryptic nature in their life styles. Yeast endophytes render wide advantages in agribusiness sector compared to filamentous fungi keeping in view their simpler cultivation and application technique. They also outshine bacterial endophytes in their capability to be stored as freeze dried and more easily dispatched for use in agricultural purpose [1]. Being ubiquitous in their phytobiome and huge potential in agribusiness sector, endophytic yeast needs to be digged More Deeper. Yeast endophytes were characterized by Xin *et al* as: "Unicellular fungi that reproduce asexually by budding—without a hyphal phase or with a reduced hyphal phase—and can live in their host without generating apparent harm" [2]. Yeasts belonging to ascomycete and basidiomycete are known to be both endophytes and epiphytes [3]. Strong prevalent reports of basidiomycotic yeasts have been found in tissues of plants eg. Ficus plant leaves [4]. These organisms show mutualistic relationship with host plants and promote phytohormone and enzyme production, stress alleviation, defense against pathogenic microbes and increased uptake of nutrients by plants [1]. Amongst the enzymatic package, cellulose degrading yeasts renders prime role in recycling and industrial bioconversion of the cellulosic material. Further, the hydrolysed small sugar units can be employed in biofuel or bioethanol production [5].
Cellulose is known to be the most abundant and naturally occurring biopolymer that yields glucose upon acid hydrolysis [6]. Its hydrolysis takes place by three means i.e chemical, physical and enzymatic. In this context, fungal enzymes outstand in terms of the enzymatic means [7]. The enzyme package secreted by endophytic fungus can be used to hydrolyse the carbohydrate from plants and hence, represent the underlined path for cellulose screening [8].
Facts from work of Sunitha *et al*, 2013 reveals 50 species of fungal endophytes isolated from *Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum* and *Catharanthus roseus*; among which 32% exhibited secretion of cellulose [9]. Another report suggested by Devi *et al*, 2012 demonstrated potential of cellulase secreting fungal endophyte from *Centella asiatica* [10].
Lipases are hydrolases present in plants, microorganisms and animal sources that breaks down triacylglycerol into simpler forms of fatty acids and glycerol [11]. The multi-dollar potential biological industries like pharmaceutical, cosmetic, food and biomedical sciences largely depends upon the contribution of lipases [12]. Again, the microbes present themselves as rich sources of lipase enzyme; eg. *Rhodotorula* spp, *Candida* spp, *Aspergillus* spp etc [13, 14].
The present study aimed to isolate yeast endophyte from leaves of *Capsicum assamicum* and identification using ITS sequencing and also checked for its cellulolytic and lipolytic activities. To our knowledge, this is the first report of *C.calyptogenae* from leaves of *C.assamicum leaves* from Guwahati, Assam region.

Materials and Methods
Sample collection
The sample used in this study was *Capsicum assamicum*. Young, healthy and disease free leave sheaths of *C.assamicum* were freshly collected from Jatia area of Guwahati and used for isolation.

Isolation of yeast endophytes
The leaf sample was first rinsed in running tap water to remove traces of dust and debris. The samples were surface sterilized using standard protocol: distilled water, 70% ethanol, 3% NaClO. Sterile filter paper was used to blot off excess moisture. Excess moisture was soaked using tissue paper. Then the leaf segments were cut into small segments of 5-8mm and placed onto potato dextrose agar (PDA) media plate supplemented with streptomycin (10mg/10ml) and incubated at 37 degree celsius for 4-6 days [15]. The cultures evolved out of these leaf segments were isolated

and the cultures were routinely maintained on slants for further analysis.

Morphological identification using lactophenol cotton blue staining

The isolated endophytic yeast was characterized based on its colony characteristics and hyphae structures. Standard protocol of lactophenol cotton blue staining was done as per Astrid 1999 [16].

Molecular characterization of the yeast endophytic isolate

Genomic DNA isolation [17]

A small yeast mass from the endophytic culture was scraped out using a fine spatula. It was placed in a tube of 2ml having ceramic pestle, sterile glass bead (60-80mg) and lysis buffer. In a tissue homogeniser, it was homogenized twice at 6 M/S for 60 sec. Next centrifugation at 13,000 rpm was done for the yeast tissue for 10 mins. Following centrifugation, the supernatant was collected and transferred to microcentrifuge tube. An amount of 2 of RNase A (10mg/ml) was added and incubation was done at 37ºC for 15 mins. An equal volume of phenol: chloroform: isoamyl alcohol was added in the ratio of 25:24:1. It was thoroughly mixed well and again placed at 13,000 rpm for 10 mins for centrifugation. After centrifugation, the upper aqueous layer was transferred into a fresh microcentrifuge tube. To it, an equal portion of 100% chilled ethanol was added. This was allowed to precipitate at -20 ºC for 30 mins. The next round of centrifugation was performed for a period of 10 mins to pellet down the DNA. The DNA pellet was washed with 70% ethanol. Again the final round of centrifugation was done at 12,000 rpm for 5 mins. Finally the obtained DNA pellet was allowed to air dry till the evaporation of ethanol. The pellet was resuspended in 1X TE buffer and stored properly at -20 ºC.

PCR amplification and sequencing

The DNA quality and quantity was monitored on 1% agarose gel and further visualized using ethidium bromide under UV-trans-illuminator [18]. Amplification of ITS region of 18S rRNA was performed with the primers of PCR (ITS1: TCCGTRSGNGAACYTGHGG and ITS4: TCCTCCGCTTATTKATDTGC) [19]. With a final volume of 100 µL, PCR amplification was done using the conditions: DNA 1 µL, both primers 400 ng, 2.5mM each DNTPs 4 µL, 1µl of Taq DNA polymerase (3U/ µL) and 10 µL of 10X Taq polymerase assay buffer. The thermal cyclic conditions operated includes initial denaturation at 95ºC for 5min, 35 cycles at 94ºC for 30 sec, 52ºC for 30sec, and 72ºC for 45sec, and final extension at 72ºC for 7mins. The products of amplification was electrophoresed on agarose gel and using ethidium bromide under UV trans-illumination [20]. By using an automated DNA sequencer (ABI 3500 Genetic Analyzer), sequencing was done as per Big Dye Terminator version 3.1 sequencing kit (Applied Biosystem) at Chromous Biotech, Bangalore. The obtained sequences were compared using BLAST search [21].

ITS sequence and Phylogenetic analysis:

The phylogenetic tree was created using Weighbor with alphabet size 4 and length size 1000. A distance matrix is generated using the Jukes-Cantor corrected distance model [22].

Screening of the isolated fungal endophyte for cellulolytic and lipolytic activities [23, 24]

The fungal endophyte was cultivated on PDA media supplemented with Na carboxymethyl cellulose. 0.2 Aqueous congo red was used to flood the plates after incubation followed by a destain using NaCl of 1M concentration for 15 mins. The observation of clear zone around the endophytic colony indicated the cellulase activity.

For lipase activity, the endophytic isolate was cultured on PDA media supplemented with Tween 20. Formation of clear zone around the colony indicated lipase secretion by the endophyte.

Results and Discussion

A mixture of fungal endophytic colonies were observed on PDA plates upon isolation of young healthy symptomless leaves of *C.assamicum*. Keeping into account the target of our study, yeast endophyte was sub-cultured and obtained as pure culture. The morphological and growth characteristics observed on culture plate was noted. Morphological observation showed appearance of pink pigmented slimy colonies on PDA plates (fig 1a). The organism was slow growing and took almost a week to cover the plate. Streak culture appeared shiny, mucilaginous and smooth. After 10 days of growth, the cells appeared ovoid to elongated. Both ballistoconidia as well as pseudohyphae were not seen (fig 1b). These features were closely related to the genus *Cystobasidium* [25] belonging to class cystobasidiomycetes.

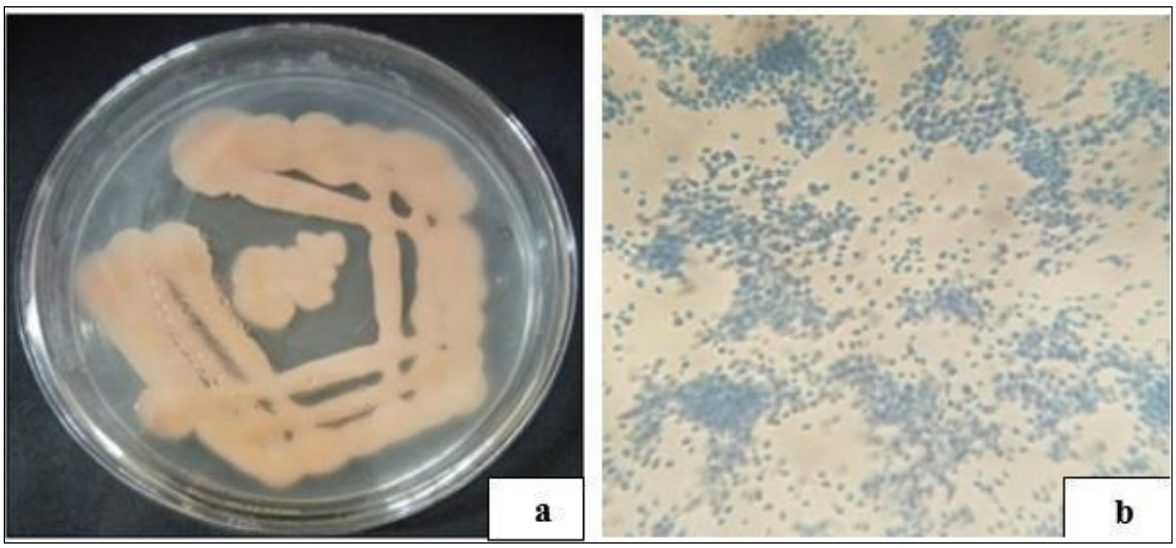

Fig 1: a) Pink pigmented slimy colony of *C.assamicum* on PDA plate **b)** Lactophenol cotton blue staining showing morphology of the yeast endophyte

The molecular approach of identification was based on the sequencing of ITS region of amplified rDNA which was further aligned with NCBI retrieved ITS regions of different organisms done by using CLUSTAL W. The endophyte's ITS sequence was checked for its most homologous sequence similarity from NCBI database. The successful ITS gene region amplification was evidenced by spotting the wells of the gel that showed single bands of the amplicons closer to 600 bp marker. Based on the BLAST analysis and phylogenetic analysis, the isolated endophyte was found to be *Cystobasidium calyptogenae* strain UA78 belonging to genus Basidiomycota and class Cystobasidiomycetes which has 99% sequence similarity with *Uncultured fungus clone S46T_49* (fig 2). Studies by Yurkov *et al* [26] suggests that genus *Cystobasidium* was previously classified as *Rhodotorula*. Reports from another study revealed the presence of *Rhodotorula* (now *Cystobasidium*) from leaves of healthy apple trees (*Malus domestica*, BORKH.) existing as endophytes [27] which is in accordance to our study. Also similar studies by Tsuji M *et al* [28] isolated four yeast colonies which were light pinkish in color and identified as *Cystobasidium tubakii* sp.and *Cystobasidium ongulense* sp., thus correlating the colony characteristics at the genus level with our study.

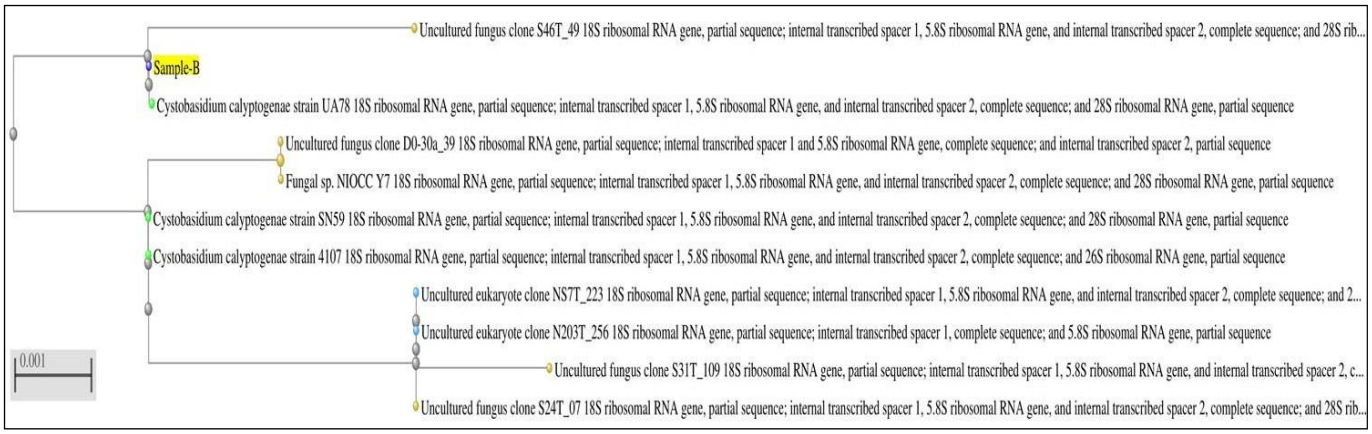

Fig 2: Phylogenetic tree formed by Weighbor joining method using ITS sequence of yeast endophyte *C.calyptogenae* and related fungi

C.calyptogenae also exhibited good potential source of cellulolytic and lipolytic activities. The yeast colony showed a defining clear zone around the colony on PDA plate supplemented with Tween 20 (fig 3a). Similar lipase secretion activity with clear zone was observed in another study done on *Candida guilliermondii*, a yeast endophyte obtained from leaves of castor (*Ricinus communis* L.) which was further processed for oleic acid esterification for potential use in production of biodiesel [29]. In terms of cellulose secretion, *C.calyptogenae* exhibited distinct clear zone around their colony upon staining using congo red which clearly indicates CMC degradation which can be clearly attributed to the presence of cellulose activity (fig 3b). Methods used previously employed use of crystalline cellulose where slow rates of degradation limited its use and thus, later assays switched to carboxymethylcellulose (CMC) which turned out to be better in terms of solubility and degradation [30]. Therefore, extracellular production of cellulose by endophytic fungi and yeast were often plated on PDA plates supplemented with the substrate, CMC [31].

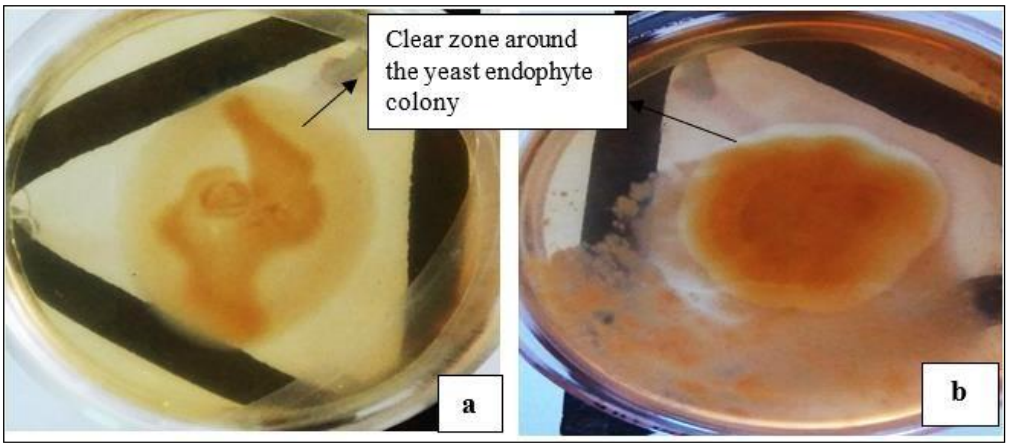

Fig 3: *C.calyptogenae* showing a) Lipolytic activity and b) Cellulolytic activity

Conclusion

It was seen that *C.assamicum* leaves from Guwahati, Assam region, first reported the presence of *Cystobasidium calyptogenae*, a yeast endophyte. Interestingly this endophytic species also proved as potential candidates for secretion of cellulase and lipase enzymes, which also is reported for the first time. This further requires quantification assays and opens up new avenues for its use in various industrial sectors.

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Conflict of Interest

The authors declare no conflict of interest.

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