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Exopolysaccharide Production Potential of Yeast *Cryptococcus flavescens SK01* Strain from the Phylloplane of *Semecarpus kathalekanensis*

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A yeast strain (SK01) was isolated as part of a study of phylloplane yeast flora of *Semecarpus kathalekanensis,* an endangered forest tree from Myristica swamps of the western ghats of Karnataka employing standard methodology. It was characterized as *Cryptococcus flavescens* based on phenotypic and molecular (rDNA) analyses. Further its exopolysaccharide (EPS) production potential was characterized. It was found to produce 4.7g.L^{-1} (on dry weight basis) of crude EPS with gross chemical composition: 88.5%, 6.15% and 5.35% of total carbohydrates, proteins and ash contents respectively which indicate its reasonably good purity. Present exercise underscores the importance of yeast diversity studies in under-explored natural habitats, *e.g.* tropical plant phylloplane.

Key words: Exopolysaccharide, phylloplane, yeasts, tropical forest.

Polysaccharides – polymers of simple carbohydrates are incorporated into the products of a variety of industries – food, cosmetics and pharmaceuticals etc. to impart desired rheological behavior to the products. Traditionally plants and algae have been the most important sources for industrial polysaccharides. An important provenance for industrial polysaccharides other than plants and algae is the Microbial Exopolysaccharides (EPS). A wide variety of microbes - both of prokaryotic and eukaryotic affinities - found in/on various natural substrates possess the ability to produce these polysaccharides external to their cellular boundaries. Microbial EPSs oftentimes possess unique structures and composition and as a consequence, exhibit unique rheological properties that may qualify them as readymade candidates for incorporation in products. Among microbial EPSs, yeast EPS production systems have been favored over bacterial EPSs for practical reasons (Petersen *et al.*, 1989, Petersen *et al.*, 1990). This has spurred interest in yeast EPSs and provided impetus for isolation of EPS producing yeasts from natural substrates and characterization of their EPS production kinetics and also structure and compositions of respective EPSs.

Phylloplane, one of the largest terrestrial habitats on the earth (Morris 2001), considered as an extreme habitat (Fonseca and Inacio 2006) frequently harbor yeasts with the ability to produce EPS whose purported utility for their producers is, as matrix of embedment on leaf surface and as means of overcoming a variety of stresses (eg.

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desiccation stress) (Vorholt 2012). Yeast species belonging to genera *Aureobasidium, Bullera, Candida, Cryptococcus, Hansenula, Lipomyces, Rhodotorula, Sporobolomyces, Tremella* and *Trichosporon* that can potentially occur in the phylloplanes (Fonseca and Inacio 2006) have been reported to produce EPSs such as mannans, phosphomannans, galactomannans, glucomannans, and glucuronoxylomannans (De Baets *et al.*, 2002). Present communication reports the characterization of EPS production potential of the yeast *Cryptococcus flavescens* isolated from the phylloplane of *Semecarpus kathalekanensis*.

MATERIALS AND METHODS

Isolation and characterization of SK01Yeast

Yeast SK01 was isolated from washes of leaves of *Semecarpus kathalekanensis* (an endangered forest tree from Myristica swamps of Kathlekan forest of Siddapur Range in Uttara Kannada district of Central Western Ghats, Vasudeva *et al.,* 2001) spread over YM (Yeast extract-Malt extract) agar (Yeast extract 0.3%, Malt extract 0.3%, Peptone 0.5%, Dextrose 1% and Chloramphenicol 200ppm). The yeast was purified and subjected to characterization as per the methodology of Yarrow (1998). Also, a partial sequence of its rDNA spanning ITS regions and a contiguous 5' region of 26S rRNA gene was characterized by Sanger sequencing method (outsourced to a local service provider) using the primer pair ITS-1 and NL4 (Kurtzman and Robnett 1998) after amplifying the region using the same primer pair. A visually corrected contig (1,058b) of forward sequence read and the reverse complement of reverse sequence read was compared to dynamic, non-redundant database of nucleic acid sequences maintained at GenBank (Benson *et al.*, 2013) using BLAST tool (Altschul *et al.*, 1990) accessed at www.ncbi.nlm.nih.gov. Further a consensus phylogeny of *Cryptococcus* species was reconstructed from a multiple alignment (using ClustalW implemented in MEGA v5.2, Tamura *et al.,* 2011) of contig rDNA sequence of SK01 and homologous (contiguous stretch of ITS and 26S D1/D2) partial rDNA sequences (retreived from GenBank, Benson *et al*., 2013) of *Cryptococcus* species, using Neighbor joining algorithm implemented in MEGA v5.2 (Tamura *et al.*, 2011) and the same was tested by bootstrapping (1000 replications).

Characterization of EPS production potential

To evaluate SK01 strain of *C. flavescens* for EPS production potential, crude EPSs were extracted from the cell free culture broths of the same, following the methodology of Grigorova *et al* (2004). It involved inoculating (@ 5%) SK01 cells into 47.5ml of EPS medium in 250ml Erlenmeyer flasks (Sucrose 5.0%, $(NH_4)_2SO_4$ 0.25%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.01%, $CaCl_2 \cdot 2H_2O$ 0.01%, and yeast extract 0.1%, Grigorova *et al.*, 1999) drawn from 48hr old cultures of SK01 strain in 2.5ml of production medium, and subsequent incubation at 28°C for 6 days on a rotary shaker rotating at a speed of 200rpm. At the end of incubation, cells were separated from culture broth by centrifugation at 6000*g* for 30 minutes. Cell free culture broth was transferred into separate 250ml Erlenmeyer flasks and added with 2 volumes (150ml) of 96% ethanol and incubated for 24hours at 4°C, to precipitate out EPSs. EPSs were later pelleted by centrifugation of cell free culture broths at 5000*g* in pre-weighed oakridge centrifuge tubes, while discarding supernatants. EPS pellets were then washed with ethanol, dried, weighed and stored (crude EPS). Meanwhile cells pellets from first centrifugation were washed twice with distilled water and dried in pre-weighed tubes at 80°C to a constant weight. Total carbohydrate content, protein content and ash content of SK01 EPS was determined by Phenol-Sulfuric acid method of Dubois (Dubois 1956), Folin's Phenol Reagent Method (Lowry *et al.*, 1951) and gravimetric method after calcination respectively. The experiment was conducted with five replications starting from inoculum preparation to EPS isolation and further analysis.

RESULTS AND DISCUSSION

Characterization of SK01isolate

SK01 yeast grew as glossy, creamish white colored colonies (Fig 1) on YM agar and had spheroidal cell morphology (Fig 2) with dimensions 2.0 -5.2 × 3.0 - 6.0 µm, determined by micrometry. It lacked fermentation ability, but was positive for both Diazonium Blue B reaction and urea hydrolysis tests. It exhibited assimilation profiles of carbon and nitrogen compounds as in Table 1. In the

comparison of SK01 rDNA contig sequence to the GenBank nucleotide sequence database by BLAST search, SK01 rDNA sequence matched with that of *Cryptococcus flavescens* and shared >99% identity at an E-value 0.0. In the consensus phylogeny reconstruction (Fig 3), SK01's partial rDNA sequence clustered with those of *Cryptococcus flavescens* (from elsewhere) in 99% of bootstrap replications. Based on phenotypic characters and rDNA analyses, SK01 yeast isolate was identified as a strain of *Cryptococcus flavescens*.

EPS production potential of *Cryptococcus flavescens* SK01

Averaged dry weights of biomass and EPS, carbohydrate, protein and ash contents of *Cryptococcus flavescens* SK01 EPS are as in the Table 2. It produced 235.1(S.D. 1.23) mg of crude EPS for a corresponding average of 294.64 (S.D. 1.53) mg of dry biomass per 50ml EPS production medium. In other words this strain was able to produce 4.7g.L^{-1} @ ~80g of crude EPS for every 100g of dry biomass. Previously, strains of other two species of *Cryptococcus*: *C. flavus* AL51 (Pavlova *et al.*, 2009) and *C. laurentii* AL100 (Pavlova *et al.*, 2011) have been reported to produce EPS of heteropolysaccharide type at 5.75g.L^{-1} and 6.4g.L^{-1} respectively on the same production medium. However those strains were from Penguin feathers and the Antarctic soil respectively. There have been no such reports of EPS production potential either of *Cryptococcus* or of any other yeast from tropical phylloplane habitats except for *Aureobasidium* isolates (Prasongsuk *et al.*, 2005) which produce EPS of homopolysaccharide type. Total carbohydrate content, protein content and ash contents (88.5%, 6.15% and 5.35% respectively) of *Cryptococcus flavescens* SK01 are comparable to the EPSs from *C. flavus* AL51 (92.5%, 3.34% and 4.16%

Table 1. Carbon and Nitrogen compound assimilation profile of *C. flavescens* SK01

D-Glucose	+	Melezitose	+
D-Galactose	+	Inulin	-
L-Sorbose	-	Starch	-
D-Glucosamine	-	Glycerol	-
D-Ribose	+	Erythritol	-
D-Xylose	+	Ribitol	+
L-Arabinose	+	D-Glucitol	+
D-Arabinose	+	D-Mannitol	+
L-Rhamnose	+	Galactitol	+
Sucrose	+	myo-Inositol	+
Maltose	+	D-Gluconate	+
α,α-Trehalose	+	DL-Lactate	d
α-Methyl-D-Glucoside	+	Succinate	+
Cellobiose	+	Citrate	+
Salicin	+	Nitrate	-
Melibiose	+	Nitrite	-
Lactose	+	Ethanolamine	+
Raffinose	+	L-Lysine	+

('+' assimilated; '-' not assimilated; 'd' delayed assimilation after 2 weeks)

Table 2. Biomass, crude EPS yield and chemical composition of crude EPS, of *C. flavescens* SK01

Dried cell biomass (mg/50ml)	Dried Crude EPS (mg/50ml)	Total carbohydrate content of EPS (%)	Protein content of EPS (%)	Ash content (%)
294.64 ± 1.53	235.1 ± 1.23	88.5 ± 1.92	6.15 ± 0.8	5.35 ± 3.8

respectively, Pavlova *et al.*, 2009) and *C. laurentii* AL100 (79.1%, 11.8% and 9.2% respectively, Pavlova *et al.*, 2011). This indicates that EPS of *C. flavescens* SK01 is of reasonably good purity, intermediate to those of *C. flavus* AL51 and *C. laurentii* AL100. This notwithstanding, thorough investigation of rheological properties of *C. flavescens* SK01 EPS is necessary as only this decides its potential application in different areas.

Fig. 1. *C. flavescens* SK01 colonies (marked with rings) on YM agar isolation plate

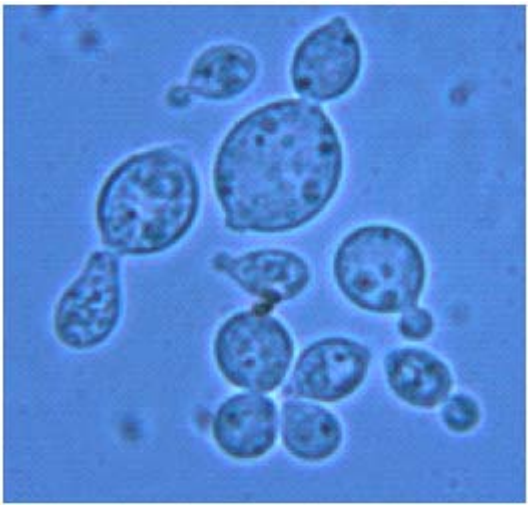

Fig. 2. Light Microscopic view (100×) of *C. flavescens* SK01

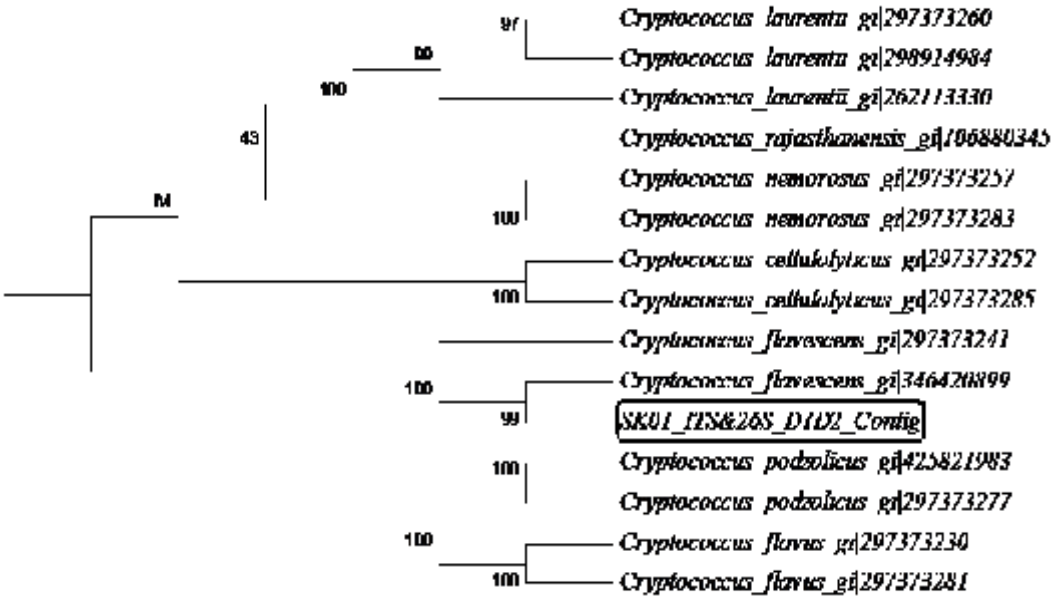

Fig. 3. Consensus Neighbor joining phylogenetic reconstruction depicting clustering of partial rDNA sequence of SK01 with those of other *Cryptococcus* species from GenBank (Benson *et al*., 2013). Numbers at the nodes indicate percentage of bootstrap replications, the respective clades remained together

CONCLUSION

With the possibility of improving the yield of EPS further than 4.7g.L^{-1}, through optimization of production parameters (*e.g.* temperature and P^H) and considering the purity of its EPS, *Cryptococcus flavescens* SK01 can be a potential candidate for development into a commercial strain for EPS production. Considering the increasing demand for novel sources of polysaccharides (either known or novel), one of the likely natural habitats of yeasts with EPS producing ability to look for could be tropical forest plant phylloplane, as they are underexplored with respect to fungal diversity, let alone yeast diversity (Hawksworth 1991). In this context present exercise of preliminary characterization of EPS production potential of a phylloplane yeast assume significance.

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