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Screening and studying enzymatic activity of cellulase producing yeasts

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ABSTRACT

Forty nine isolates of cellulose-degrading yeast were isolated from different sugar factories enriching by using the carboxymethyl cellulose agar medium (CMC). To check the cellulase activity of the organisms diameter of clear zone around the colony and hydrolytic efficiency on cellulose were measured by Congo red assay. Among the yeast isolates CYLL21 and CYLL32 exhibited the maximum zone of clearance around the colony with diameter of 28 and 26 mm and the cellulolytic efficiency of 280 and 250 per cent respectively. The extracellular cellulase activity ranged from 22.0 to 17.83 U/ml in 48 h at 35°C for yeast isolates.

Keywords: Cellulose degrading yeast; CMC activity; pH; carbon source; temperature

INTRODUCTION

Cellulase is an important extracellular microbial enzyme which hydrolyzes cellulose that is the most inexpensive source of biomass utilized for the production of valuable components such as sugar, protein and other chemicals via enzymatic bioconversion and hence this enzyme has high industrial value ((Lynd et al 2002). Cellulose which forms almost half of the dry weight of the earth's biomass is an un-branched polymer consisting of □-glucose units linked by 1, 4-□ glycosidic bonds (Fengel and Wegener 1984). This macromolecule has a complex crystalline structure, is insoluble in water and is quite resistant to depolymerizing enzymes and chemical reagents. Cellulose degrading enzymes are secreted by microorganisms into the surrounding environment and as with most enzymes degrading biopolymers they constitute a multi-component lytic complex that acts synergistically on the cellulose macromolecule (Susan 1995). Cellulase enzymes have various applications like breakdown of plant walls (cellulose), digestion of fiber, fermentation, drug withdrawal, cell detox, colon cleaning and pain syndromes, Candida (yeast infections), gas, bloating, acute food allergies, facial pain or paralysis, food

processing, poultry and textile industry (Bhat 2000). Most of the research work is concentrated on the cellulose decomposing bacteria and fungi. However very few studies reported the use of yeast for decomposition of cellulose. In the present study isolation and screening of cellulolytic yeast from Tamil Nadu sugar factories and their enzymatic hydrolysis are reported

MATERIAL and METHODS

Collection of samples

The samples collected from different sources were sludge biomass, spent sludge, spent liquid, spent wash, molasses, compost, pressmud and soil samples from M/s Kothari Sugars and Chemical Limited (Ariyalur and Laigudi, Trichy, Tamil Nadu), M/s Rajashree Sugars and Chemicals Limited (Vagai Dam, Theni, Tamil Nadu), M/s Sakthi sugar mills (Sivagangai, Tamil Nadu) and EID Parry (Aranthangi, Pudukottai, Tamil Nadu). The samples were collected in sterile NASCO (102 micron) polybags, transported to the laboratory and stored at 4°C until analysis.

Isolation of cellulolytic yeast

Isolation of yeast isolates was done by an enrichment technique using the carboxy methyl cellulose broth (CMC). One gram of sample was aseptically placed in 250 ml Erlenmeyer flask containing 50 ml enrichment broth and incubated at 32 ± 2°C in an incubator cum shaker at 120 rpm for 48 h. A loopful of the enriched culture was streaked on CMC agar medium and incubated at 32 ± 2°C until yeast colonies appeared. Morphologically distinct, single and well-separated colonies from each sample were randomly picked up and purified by repeated streaking on the CMC agar plates and incubated at 32 ± 2°C for 48 h. Purified yeast strains were maintained on YPD agar (yeast peptone dextrose agar), CMC and NA (nutrient agar) slants and stored at 4°C and subcultured at regular intervals in order to maintain the viability. Stock cultures of all the isolates were preserved in 50 per cent glycerol at -20°C.

Screening of cellulolytic yeast

In order to screen an efficient cellulose degrading yeast the cellulase efficiency was tested in plate assay using the CMC medium. Yeast isolate 10 µL (1 x 10^7cfu/ml) was spotted at CMC agar plate and incubated at 30°C for 3 days. At the end of incubation hydrolysis zone was visualized using the Congo red assay. To determine the cellulase activity of the yeast diameters of clear zone around the colony and colony diameter were measured. The experiment was repeated three times and results are expressed as cellulase efficiency= clear zone diameter/colony diameter x 100 (Teather and Wood 1982).

Identification of cellulolytic yeast

The selected yeast isolates were characterized based on their cell morphology viz cell shape, size, arrangement, budding pattern and growth

in liquid medium and colony characteristics like shape, elevation, size, texture, appearance and pigmentation. Biochemical characterization of yeast isolates was also carried out which determines the ability of yeast to ferment different carbon sources.

Enzyme assay

Total cellulose activity was determined by measuring the amount of reducing sugar released from filter paper. Endoglucanase (□-1-4 endoglucanase-EC 3.2.1.4) activity was assayed by measuring the amount of reducing sugar released from CMC. Endoglucanase activity was determined by incubating 0.5 ml of supernatant with 0.5ml of 2 per cent CMC in 0.05 M sodium citrate buffer (pH 4.8) at 50°C for 30 min. After incubation for an hour at 50°C the reaction was terminated by addition of 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent to 1 ml of reaction mixture. In these tests reducing sugars were estimated spectrophotometrically (Shimadzu, UV- 160, Japan) at 575 nm (Miller 1959) using glucose as standard. The enzymatic activity of total endoglucanase is defined in international units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µ mol reducing sugars (measured as glucose) per ml per minute (Irfan et al 2012).

Effect of temperature on cellulase activity

The isolated strains were inoculated on CMC broth and incubated at different temperatures (25, 35, 45 and 55^{o}C) for 3 days separately. After the end of incubation the cellulase activity was checked as described previously (Miller 1959). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per ml per minute.

Effect of pH on cellulase activity

A set of CMC media was prepared with varying pH (3.5, 4.5, 5.5, 6.5 and 7.5). Media were autoclaved and inoculated with yeast isolates. Flasks were incubated at 30^{o}C in a rotary shaker at 120 rpm. Culture filtrate was collected after 3rd day of incubation and cellulase enzyme activity was determined.

Effect of different carbon sources on cellulase activity

The selected yeast isolates were inoculated on basal CMC broth devoid of CMC with addition of following carbon sources (1% w/v) viz arabinose, galactose, mannose, cellulose and xylose and these were incubated at 30 ± 2°C for 3 days. Simultaneously control was kept CMC as carbon source. After the end of incubation the crude enzyme was separated and cellulase activity was measured.

Statistical analysis

All the experiments were performed in triplicates. Mean and standard deviation (SD) values were calculated using MS excel spreadsheet software. The data were analyzed with ANOVA and LSD for

tests of significance using AGRESS software.

RESULTS and DISCUSSION

Isolation and screening of yeast strains

A total of 14 samples were collected from different locations of sugar factories for isolation of yeast. From the collected samples 49 yeasts were isolated by the enrichment technique. Among the 49 isolates 25 showed positive results on cellulolytic efficiency with isolate CYLL21 showing maximum (280%) (Table 1) cellulolytic efficiency followed by CYLL32 and SCY42 (250%).

Enzyme activity

Cellulase activities of cellulolytic yeasts isolated from sugar factories are presented in Table 2. The cellulolytic yeast SCY42 recorded the maximum cellulase activity of 22.0 U/ml followed by isolate CYLL21 (17.83 U/ml) after 48 h at 35°C and that declined further (Fig 1 & 2). Among the six different carbon sources tested the cellulase activity was found to be maximum with cellobiose (Table 3). Previously similar results were reported by various authors (Nieves et al 1997, Nagar et al 2010). Cellulase from different filamentous fungi such as *Trichoderma reesei* that currently

Tables 1. Screening the efficient cellulolytic yeast isolates by Congo red test and their celulolytic efficiency

Isolate	Cellulolytic efficiency (%)	Isolate	Cellulolytic efficiency (%)
EPY11	120.0 ± 2.56	EMY41	150.0 ± 1.63
EPY12	100.0 ± 2.55	EMY42	120.0 ± 2.62
EPY13	98.0 ± 0.56	EMY43	135.0 ± 1.61
EEIY21	180.0 ± 0.31	EMY4	142.0 ± 3.25
EPY21	200.00 ± 4.0	EPY51	123.5 ± 1.53
CYLL21	250.0 ± 3.25	EPY52	96.8 ± 0.75
EPY31	100.0 ± 0.05	EPY53	94.0 ± 0.39
EPY32	115.8 ± 0.06	EMY51	120.0 ± 1.43
EPY33	122.0 ± 1.52	EMY52	125.0 ± 1.75
EPY34	130.0 ± 2.57	EBY 61	115.8 ± 2.10
EPY35	132.0 ± 0.96	EBY62	120.0 ± 2.06
CYLL32	280.8 ± 2.63	EPY61	135.0 ± 2.51
CYLL31	126.0 ± 3.10	EPY62	128.0 ± 1.66

SEd= 3.11, $CD_{0.05}$= 6.24

Values are means of three replications of ± SD (standard deviation)

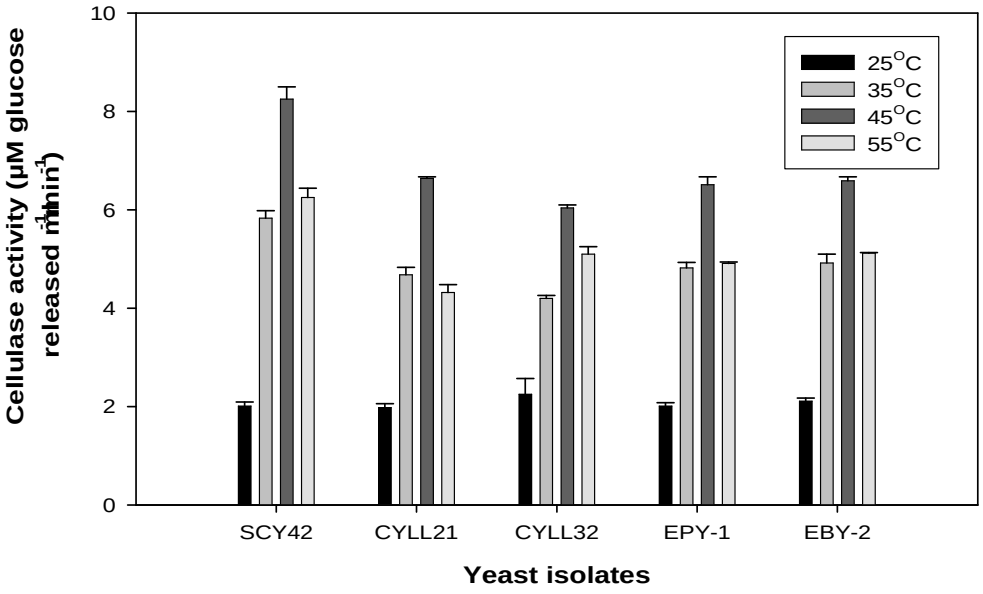

Fig 1. Cellulase activity of yeast isolates at different temperatures

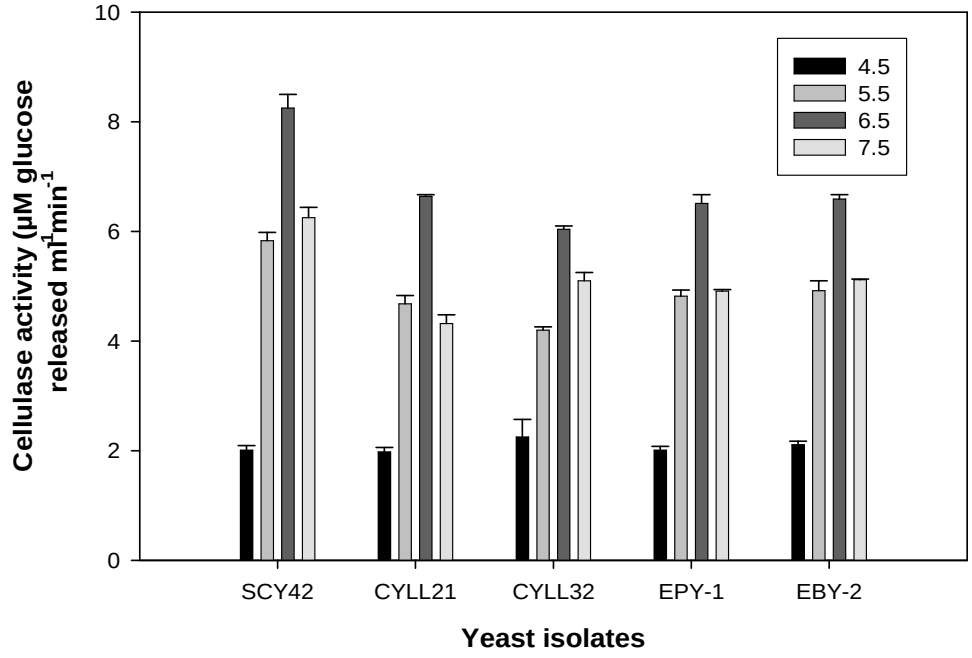

Fig 2. Cellulase activity of yeast isolates at different pH levels

Table 2. Cellulase activity of selected yeast isolates

Isolate	Cellulase activity (U/ml)	Isolate	Cellulase activity (U/ml)
EEIY	14.67 ± 0.106	CYLL21	17.83 ± 0.176
EBY21	14.67 ± 0.328	CYLL32	12.57 ± 0.104
EPY32	13.67 ± 0.064	EROPY12	12.43 ± 0.077
EPY43	14.17 ± 0.228	EMY63	12.67 ± 0.250
EPY62	15.17 ± 0.363	SCY42	22.00 ± 0.114

SEd= 0.29, $CD_{0.05}$= 0.61

Values are means of three replications of ± SD

dominates the industrial application of cellulase is short of the □-glucosidase activity (Brethauer and Studer 2014). Similar observations were made by Ramalingam and Ramasamy (2013).

Identification of cellulolytic yeast

The yeast colonies were whitish to creamish colour with flat to raised surface. The cell shape varied from oval, ellipsoidal to spherical with small to medium size. All the yeast isolates were found to ferment glucose, sucrose, lactose, maltose, galactose, xylose, cellulose and mannose at 10 per cent concentration (Table 4). However the isolates CYLL21, CYLL32, SCY42 and EBY showed positive result towards xylose (5 carbon sugar) and carboxy methyl cellulose (6 carbon sugar) only. The positive results for fermentative ability of the yeast isolates were identified by the formation of gas bubble and turbidity of the medium.

In the present study cellulose degrading yeasts which exhibit maximum cellulase were documented. Many of the research works are focused on isolation of cellulose degrading bacteria and fungi. Fermentation of cellulose with bacteria or fungi has been examined previously. However these conventional methods have economic disadvantages such as the production of low amount of cellulase and have not been commercialized yet. To overcome these problems many researchers have been concentrating on yeast which produces cellulases and □-glucosidase for direct fermentation.

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Table 3. Cellulase activity of yeast isolates at different carbon sources

Isolate	Cellulase activity (µM glucose released/ml/min)						
	Arabinose	Cellulose	Cellobiose	CMC	Galactose	Mannose	Xylose
EEIY	2.25 ± 0.013	10.50 ± 0.148	12.17 ± 0.306	11.33 ± 0.266	14.67 ± 0.129	8.67 ± 0.040	17.83 ± 0.341
EBY	2.02 ± 0.045	11.00 ± 0.155	11.00 ± 0.275	10.33 ± 0.124	14.67 ± 0.084	5.33 ± 0.067	12.57 ± 0.052
SCY42	2.08 ± 0.005	10.33 ± 0.054	12.17 ± 0.108	10.50 ± 0.246	13.67 ± 0.235	3.50 ± 0.042	12.43 ± 0078
CYLL21	2.13 ± 0.032	10.50 ± 0.131	12.33 ± 0.231	11.00 ± 0.246	14.17 ± 0.192	6.83 ± 0.053	12.67 ± 0.013
CYLL32	1.98 ± 0.001	9.33 ± 0.189	13.00 ± 0.311	11.17 ± 0.035	15.17 ± 0.008	8.83 ± 0.055	22.00 ± 0.286
CYFS2	2.00 ± 0.021	10.50 ± 0.016	12.17 ± 0.222	10.83 ± 0.242	14.33 ± 0.045	8.33 ± 0.091	12.65 ± 0.119
SEd	0.035	0.184	0.355	0.298	0.197	0.085	0.271
$CD_{0.05}$	0.076	0.401	0.775	0.649	0.431	0.186	0.591

Values are means of three replications of ± SD (standard deviation)

Table 4. Biochemical test for selected yeast isolates

Biochemical test	EEIY	EBY21	EPY32	EPY43	EPY62	CYLL21	CYLL32
Fermentation of carbohydrates							
a. Glucose	+	+	+	++	++	+	++
b. Sucrose	+	+	+	+	++	+	+
c. Lactose	-	-	+	-	+	-	+
d. Maltose	+	+	+	+	+	+	+
e. Xylose	+	+	+	+	+	+	+
f. Mannose	-	-	-	+	+	+	-
Acid production from glucose	-	-	-	-	-	-	-
Urease activity	+	+	+	-	-	+	+
Gelatinase activity	+	-	-	-	-	+	-
Sporulation	+	+	+	+	+	+	+
Tentative identification	*Saccharomyces* sp	*Saccharomyces* sp	*Saccharomyces* sp	*Saccharomyces* sp	*Saccharomyces* sp	*Saccharomyces* sp	*Saccharomyces* sp

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