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Isolation and Characterization of Cellulose Degrading *Candida tropicalis* W2 from Environmental Samples

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Abstract.- Microbial cellulose degradation is significant in environment rich in plant organic matter. The present study was carried out to isolate cellulose degrading yeasts from environmental samples. The isolated yeast characterized through 18S rRNA was found to be *Candida tropicalis*. *C. tropicalis* showed optimum growth at 37°C and pH of 7. The isolated yeast produced both intra-as well as extra-cellular enzyme but the dominant form found was extra-cellular. *C. tropicalis* showed maximum enzyme production after 5 days of incubation. The optimum temperature and pH for cellulase activity was found to be 40°C and 8, respectively. Cellulase activity was inhibited in the presence of all tested metal ions. *C. tropicalis* can be exploited for cellulose biotechnologies.

Key Words: Carboxymethyl cellulose, cellulase, *Candida tropicalis*, cellulose degradation

INTRODUCTION

In recent years it has been proposed that instead of traditional feed stocks (starch crops), cellulosic biomass (cellulose and hemicellulose), such as agricultural and forestry residues, waste paper, and industrial wastes, could be used as an ideally inexpensive and abundantly available source of sugar for fermentation into the sustainable transportation fuel ethanol (Fujita *et al*., 2002; Edwards and Doran-Peterson, 2012). Cellulose is deceptively simple chemically, a polymer consisting only of glucose linked only by β 1, 4 bonds. But cellulose samples of different origin vary widely in chain length and the degree of interaction between the chains (Eveleigh *et al.*, 2009). The breakdown of biomass involves the release of long-chain polysaccharides, specifically cellulose and hemicellulose, and the subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon chain sugars (Rubin, 2008).

Cellulases are inducible enzymes, which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). Microorganisms hydrolyze and metabolize insoluble cellulose by these extracellular cellulases that are either free or cell associated. The biochemical analysis of cellulase systems from aerobic and anaerobic bacteria and fungi has been comprehensively reviewed during the past two decades. Cellulases have attracted much interest because of the diversity of their application such as their usage in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness (Cavaco - Pola, 1998). A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Gong *et al*., 1999; Gujjari *et al*., 2011; Todaka *et al*., 2011).

The degradation of cellulosic biomass with the help of microbial enzymes increases their availability at a wide range which can be used industrially. There are wide varieties of bacteria and fungi that can degrade cellulose as a carbon source. It has been recognized that the microorganisms such as thermophilic aerobic fungi (*Sporotrichum thermophile*, *Thermoascus aurantiacus*, *Humicola insolens*), mesophilic anaerobic fungi (*Neocallimastix frontalis, Piromonas communis*) mesophilic and thermophilic aerobic bacteria (*Cellulomonas* sp., *Cellvibrio* sp.), mesophilic and thermophilic anaerobic bacteria (*Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens* and *Clostridium thermocellum*) as well as actinomycetes (*Thermomonospora fusca*) produce active cellulases (Bhatt *et al*., 1993). There have been many articles dealing with more efficient cellulose degrading

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enzyme from various organisms such as *Trichoderma reesei*, *Trichoderma viride*, *Trichoderma lignorum*, *Chrysosporium lignorum*, *Chrysosporium pruinosum*, *Fusarium solani* and *Cryptococcus* species, but there are only a few which have identified the yeast as cellulase producer (Lamed and Bayer, 1988).

In this study, a mesophilic strain of *C. tropicalis*, proved to be a good producer of cellulase, was isolated from environmental samples. Moreover, some of the critical factors affecting cellulase production by this strain were also optimized.

MATERIALS AND METHODS

Isolation of cellulose degrading yeasts

Soil and water samples from sites rich in decomposing plant cells i.e., fruit shop dumps and leather industry, Raiwind road, were collected. The samples were serially diluted and a dilution of 10^{-5} from each sample was plated on Yeast Extract Peptone Dextrose (YEPD) agar medium containing 1% carboxymethyl cellulose (CMC). These plates were incubated at 30°C for 24 to 48 hours. Initially ten yeast isolates that showed growth on 1% CMC were selected for further study.

Screening of yeast isolates on different substrates

Yeast isolates were grown on different 1% cellulosic substrates *i.e.*, CMC, filter paper, acid hydrolyzed saw dust, rice straw, wheat bagasse in mineral salt media. They were incubated in shaking incubator at 30°C for 4 days and their growth (in terms of optical density) was determined after every second day by spectrophotometer at 600 nm. The yeast isolates which showed good growth on these substrates were used for research work.

Determination of cellulase activity by yeast isolates

Four yeast isolates A2, B5, W2 and W3 were further screened by Congo red test in which yeast isolates grown on YEPD agar plates containing 1% CMC as a substrate for 2-3 days. The plates were stained by 0.2% Congo red for 30 minutes and were washed with 1M NaCl solution for 1 hour. Extra-cellular enzyme activity was confirmed by the formation of zone of hydrolysis around the growth and the yeast isolate showing maximum zone of hydrolysis was selected for further research work.

Morphological, biochemical and molecular characterization of the yeast isolate

For biochemical characterization the yeast isolate was tested for colony morphology, spore staining, starch hydrolysis, ester production, nitrate reduction, yeast-malt agar test, citrate utilization, acid production from glucose, ammonia from urea, fermentation of carbohydrates, and tolerance of 1% acetic acid. For physical and biochemical characterization of yeast isolate the criteria adopted by Benson (1994) were followed. For further identification, genomic DNA was isolated and the 18S rRNA gene was amplified by PCR using two general yeast 18S rRNA primers. The forward primer covers positions 142 to 163 *C. tropicalis* (ITS-5; 5′-GGAAGTAAAAGTCGTAACAACG-3′) and the reverse primer, ITS-4; 5′-TCCTCCGCTTATTGATATGC-3′), covers positions 526 to 545. PCR reaction conditions were as per Larena *et al*. (1999). The PCR product of (approx. 0.58 kb) was cleaned up using a Fermentas purification kit (#K0513). Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. Nucleotide sequence similarities were determined using BLAST (NCBI database; http://www.ncbi.nlm.nih.gov/BLAST). The 18S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

Determination of optimum growth conditions

For optimum growth of the yeast isolate, two parameters i.e., temperature and pH were considered. For determination of optimum temperature, 5 ml YEPD broth was added in 5 sets, each of three test tubes, autoclaved and inoculated with 20 µl of freshly prepared culture of yeast isolate. The four sets of tubes were incubated at 20ºC, 30ºC, 37ºC and 45ºC. After an incubation of 16 hours, the absorbance was taken at 600 nm using a LAMBDA 650 UV/Vis Spectrophotometer (PerkinElmer, USA).

For determination of optimum pH, test tubes having 5 ml YEPD broth were prepared in6 sets,

each containing 3 test tubes, and pH was adjusted at 5, 6, 7, 8, 9 and 10, then autoclaved. These tubes were inoculated with 20 µl freshly prepared culture of the yeast isolate. After incubation period of 16 hours, the absorbance was taken at 600 nm.

Effect of cellulosic material on yeast growth

The growth behavior of the yeast isolate was checked over a time period of 32 hours. *C. tropicalis* was grown in different media at 37°C and pH of 7. The YEPD broth was used as control and mineral salt medium with 1% different substrates (CMC, wheat bagasse, saw dust hydrolyzed) as a carbon source. The flasks were inoculated with 16 hours old yeast culture and were incubated at 37°C. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20, 24, 28, and 32 hours. Absorbance was taken at 600 nm.

Determination of cellulase activity

The yeast isolate was grown in mineral salt medium containing 1% saw dust hydrolyzed as a carbon source. The flasks were inoculated with 16 hours old yeast culture and were incubated at 37°C for 5 days. Yeast cells were harvested by centrifugation at 4000 rpm for 10 minutes. Cells were disrupted by sonication (Heilscher Ultrasonic Processors UP400, S) at 4°C for 1 time for 15 s and centrifuged at 10000 rpm for 10 minutes at 4°C. The cell lysate and culture supernatant (for extracellular enzyme activity) were saturated with 60% ammonium sulfate to precipitate the enzyme. After 24 hours, the precipitated solutions were centrifuged for 10 minutes at 10000 rpm at 4°C. Ammonium sulfate precipitated samples were desalted and cellulase activity was measured according to Cohen *et al.* (2005) by using 1% w/v CMC solution into 0.1mol/l phosphate buffer (pH 7) as a substrate. The reaction mixture contained 0.5ml of supernatant as an enzyme. The mixture was incubated at 50°C for 30 minutes. The CMC enzyme-mixed solution reaction was stopped by adding 1ml of DNS reagent followed by boiling at 90°C for 10 minutes. The developed color was read at 540 nm using spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme releasing reducing sugar equivalent to 1 µmol glucose per minute under the assay condition. Protein concentration was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Effect of pH, temperature and metal ions on cellulase activity

The effect of pH on the activity of cellulase was measured by incubating 0.5 ml of the diluted enzyme and 0.5 ml of buffers of different pH [sodium acetate buffer (pH 5-6), sodium phosphate buffer (pH7-8) and tris-HCl buffer (pH 9)] ranging pH from 5 to 9, containing 1% CMC. The effect of temperature on the enzyme activity was determined by performing the standard assay procedure (Cohen *et al*., 2005) at pH 7.0 within a temperature range of 30-90°C. After treatment the residual enzyme activity was assayed. The metal ion effect on enzyme activity was examined by chloride salts of various metals. Each metal was added in the reaction mixture at a final concentration of 0.1 mM and cellulose activity was determined at 50°C and pH 7. No metal ions were added in the control assay.

RESULTS

Isolation and screening of yeasts

Initially ten yeast isolates were selected that showed fair growth on 1% CMC, designated as A2, B2, B3, B5, B6, B7, W1, W2, W3 and W4. Four yeast isolates A2, B5, W2 and W3 showed growth on all 1% cellulosic substrates *i.e*., CMC, filter paper, acid hydrolyzed saw dust, rice straw, wheat bagasse in mineral salt medium were screened for further research work. The yeast isolates not only showed maximum growth on these substrates as the sole carbon source but also revealed that they have a pathway for the conversion of complex polysaccharides (rice starw, wheat bagasse and sawdust) into monosaccharides and then to utilize them as a carbon source. Growth rates were higher in glucose and sawdust containing media when compared with other carbon sources. The optical density was 2.0 in YEPD, 1.8 in sawdust, 1.6 in CMC and 1.5 in wheat bran indicating the ability of *C. tropicalis* W2 to utilize various cellulosic materials as a carbon source as shown in growth curves experiment. Minor differences in growth

rates were observed when yeast cells were grown in wheat bagasse and sawdust containing medium.

Extra-cellular enzyme activity by C. tropicalis

Four yeast isolates A2, B5, W2 and W3 were further screened by Congo red test in which yeast isolates grown on YEPD agar plates containing 1% CMC as a substrate. Extra-cellular enzyme activity was confirmed by the formation of zone of hydrolysis around the growth when stained with Congo red and washed repeatedly with 1M NaCl solution. Among the four yeast isolates W2 showed maximum zone of hydrolysis and was selected for further research work (Fig. 1).

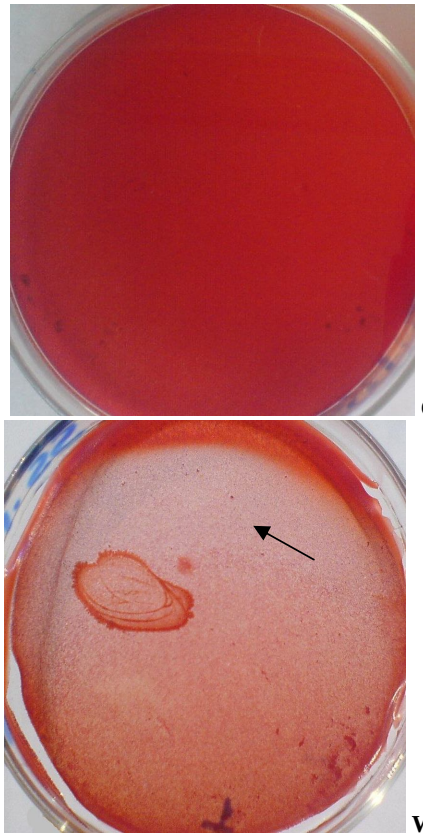


Fig. 1. Screening of cellulose degrading yeasts by 0.1% Congo red test. A2, B5, W2 and W3 yeast isolates with cellulase activity exhibited clear zones respectively. W2 isolate showed best zone so it was selected for further research.

Identification of yeast isolate

The morphological and biochemical characteristics of the yeast isolate are shown in Table I. The partially amplified (580bp) and sequenced 18S rRNA gene from local isolate (W2) was uploaded to the NCBI (National Center for Biotechnology Information) website to search for similarity to known DNA sequences in order to confirm the species of the locally isolated yeast. The BLAST query revealed that this gene is 91% homologous to already reported gene of *Candida tropicalis* (EF151451). The nucleotide sequences coding for the 18S rRNA gene of *C. tropicalis* have been submitted to the GenBank database under accession number JN009855.

Table I.- Morphological and biochemical characteristics of the yeast isolate.

Characters	***C. tropicalis***
Colony shape	Round
Size	0.1-0.36 mm
Colour	Cream
Texture	Smooth
Margin	Entire
Elevation	Raised
Type	Budding
Starch hydrolysis	-
Ester production	+
Citrate utilization	-
Tolerance of 1% acetic acid	-
Acid production from glucose	-
Production of ammonia from urea	+
Nitrate reduction	+
Sugar fermentation	+
(Glucose, Sucrose, Maltose)	+
	+

(+) positive; (-) negative

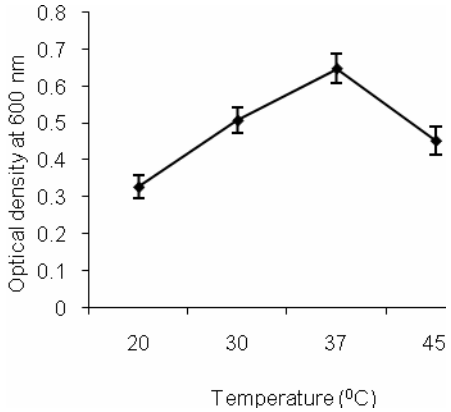
Optimum growth conditions

The most suitable temperature for the growth of purified yeast isolate (Fig. 2) was found to be 37°C. Maximum growth for *C. tropicalis* was observed at pH of 7. Two parameters (Temperature and pH) were optimized for the maximum growth of yeast cells. In the present study *C. tropicalis* is classified as mesophilic and neutrophilic yeast because showing its optimal growth at 37°C and pH of 7, respectively.

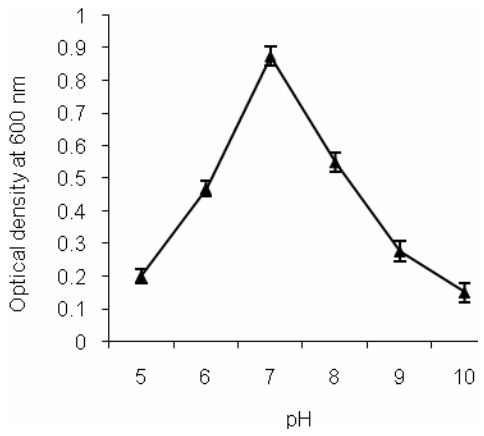
Effect of cellulosic materials on yeast growth

The growth curve pattern was studied by growing the *C. tropicalis* in the presence of 1%

cellulosic substrates (CMC, wheat bagase, and saw dust hydrolyzed, respectively) and compared with the control culture (YEPD medium) in which no cellulosic material was added. The isolate showed maximum growth in YEPD medium (Control). The maximum growth of yeast isolate was observed in mineral salt medium containing saw dust. Yeast isolate also showed good growth in CMC whereas wheat bran showed variable growth pattern.



A



B

Fig. 2:Effect of temperature (A) and pH (B) on the growth of *C. tropicalis.*

It is interesting to note that the lag phase of the yeast isolate was extended from 4 to 16 hours, then stationary phase was observed from 16 to 28 hours and finally decline phase was set up. Cellulose is a complex carbohydrate and not all microorganisms are able to use it for energy purpose. When no other carbon source except cellulose was provided in the culture medium then *C. tropicalis* W2 cells took some time to utilize this complex carbohydrate. The lag phase of the yeast isolate was extended as compared to normal growth pattern because this metabolic shifting to utilize cellulose slowed down the growth rate of the yeast cells. The growth pattern is shown in Figure 3.

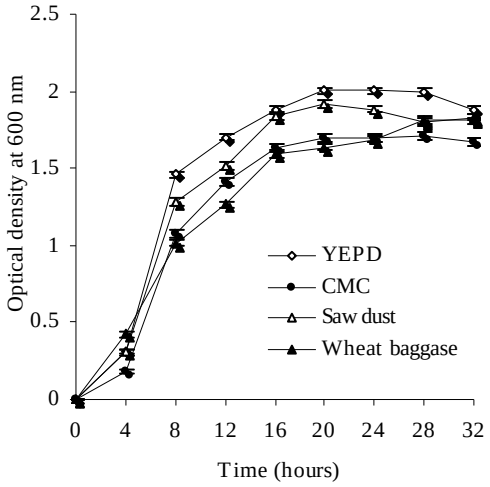


Fig. 3. Growth curves of yeast isolate in YEPD broth medium (Control) and mineral salt medium containing 1% CMC, saw dust and wheat bagasse as a source of carbon.

Determination of enzyme activity

The yeast isolate produced remarkable extra-cellular cellulase showing that it can easily degrade cellulosic substrates. The mineral salt medium contains only a trace amount of nitrogen and other minerals thus producing nutrient deficient condition with only 1% carbon source substrate. Table II shows the activity of cellulase before and after ammonium sulfate precipitation.

Intracellular versus extracellular enzyme activity

In order to determine the dominant form of the enzyme, both intra-as well as extra-cellular enzyme assay was performed. The results clearly showed that almost 50% increase was determined in case of extra-cellular in comparison with intra-cellular enzyme assay (Fig. 4). The enzymes found in the microorganisms can be of both intra as well as extra-cellular origin but in the present investigation the extra-cellular form of cellulose was dominant (Fig. 4).

Table II.- Partial purification of cellulase from *Candida tropicalis.*

Parameters	Crude enzyme	Ammonium sulfate precipitation
Amount of protein (mg)	138	89
Activity (nkat)	10,416	9,642
Specific activity (U/mg)	75	108
Purification factor (fold)	-	1.4
Yield (%)	100	92

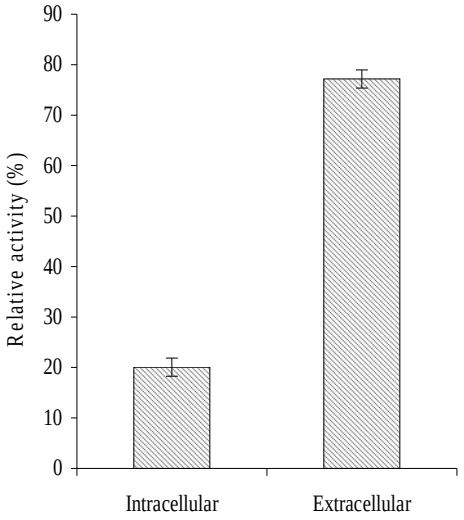


Fig. 4. Comparison between extracellular and intracellular cellulase activity of the yeast isolate.

Effect of physical parameters on enzyme activity

Effect of temperature

As temperature is one of the most important variables, when *C. tropicalis* was checked for the effect of temperature on its enzymatic activity, maximum extracellulase activity (352%) was observed at 40°C (Fig. 5A).

Effect of pH

The effect of pH on cellulase activity was also observed by incubating enzyme with buffers of different pH. The yeast isolate showed maximum enzyme activity at pH 8 (Fig. 5B). Production of cellulase by yeast isolate was found to be dependent on pH and *C. tropicalis* cellulase showed maximum activity in alkaline range *i.e.*, pH 8.

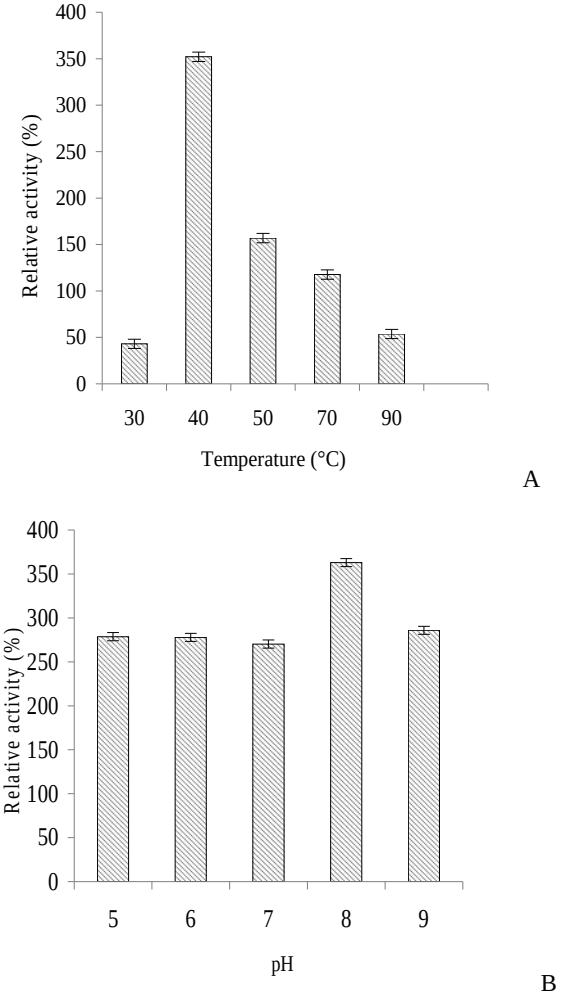


Fig. 5. Effect of temperature (a) and pH (b) on the cellulase activity of *C. tropicalis.*

Effect of different metal ions

Cellulase activity was also checked in the presence of various metal ions. The yeast isolate showed no increase in enzyme activity in the presence of any metal ion, rather it showed greatly decreased cellulase activity in the following manner; Mn^{+2} (39%), Ca^{+2} (25.1%) , Mg^{+2} (21.2%), Zn^{+2} (17.7%) , K^{+} (15%) and Hg^{+2} (13%). So it was found that the presence of metal ions inhibit the cellulase activity in *C. tropicalis.* Variable inhibition on the activity was found by the heavy metals Pb^{+2}, Cd^{+2}, Ag^{+} and Hg^{+2}. The inhibition by sulfhydryl oxidant metals (Ag^{+} and Hg^{+2}) may indicate that the thiol groups are involved in the active catalytic site.

DISCUSSION

Scientific communities have strong interests in cellulases because of their applications in industries such as starch processing, animal and food production, malting and brewing, extraction of fruit and vegetable juices pulp and paper industry, and textile industry (Kaur *et al.*, 2007; Gao *et al*., 2008). Many cellulolytic microorganisms and their enzyme systems have been studied extensively for the enzymatic conversion of cellulosic substances (Gilkes *et al*., 1991; Bey *et al.*, 2011; Boonmak *et al*., 2011). In the present investigation yeast, *C. tropicalis*, was isolated which has great potential to degrade cellulosic materials.

The cellulases are mainly extracellular enzymes produced by mesophilic or thermophilic microorganisms (Kim *et al*., 2005). Since the use of cellulose degrading enzymes is related to industrial processing and operation at high temperature, application of thermostable enzymes produced by mesophilic or thermophilic microorganisms appears to advantageous (Kim *et al*., 2005). In our study the optimum temperature and pH for the growth of *C. tropicalis* were found to be 37^{o}C and 7.

The growth pattern of *C. tropicalis* was studied in the presence of 1% CMC, wheat bagasse, saw dust hydrolyzed and yeast isolate showed maximum growth in mineral salt medium containing saw dust. The optimization of the medium for cellulase production by selecting the best nutritional and environmental conditions is important to increase the produced cellulase yield (Gomes *et al*., 2000). The lignocellulosic biomass, such as corn stover and wheat bran, are very abundant, cheap and easily available. Various agricultural substrates-byproducts and microbial cultures have been used successfully for cellulose production (Yang *et al*., 2006). In stress conditions, the organism was forced to utilize hydrolysed saw dust as the only carbon source and thus cellulase production was determined (Luo *et al*., 2009). A variety of bacterial species are able to degrade cellulose and being prokaryotes they have different optimum growth conditions. Bacterial cellulases have a broad range of pH and temperatures to degrade cellulose as comparison to yeast cellulases.

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and particularly enzyme molecules (Shah and Madamwar, 2005). The pH of the medium strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). In present study the extracellular cellulase obtained by *C. tropicalis* showed optimum activity at 40^{o}C and pH of 8. Cellulases from *Bacillus* sp. KSM-635 had an optimal activity at 40^{o}C, similar to the cellulase in the present study (Ito, 1997). The production of alkaline cellulases has been mainly reported in bacteria (Horikoshi *et al.*, 1984) but in the present study *C. tropicalis* also showed optimum activity at pH of 8.

CONCLUSIONS

In the present study a cellulose degrading yeast, *C. tropicalis*, was isolated from environmental samples. The optimal pH and temperature of *C. tropicalis* were 7.0 and 37°C, respectively. *C. tropicalis* has ability to produce cellulase both intra-as well as extra-cellular but the dominant form found to be extra-cellular. The optimum temperature and pH for the activity of cellulase were found to be 40^{o}C and 8, respectively. All metal ions have negative effect on cellulase activity. Extracellular enzyme produced by *C. tropicalis* can be exploited for cellulose biotechnologies.

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