

Optimization and Characterization of Thermostable Endo and Exocellulases by *Humicola* sp. SKESMBKU03

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Abstract	Article Information
<p><i>Humicola</i> sp. SKESMBKU03, a cellulase producer was isolated from horse dung manure collected from Hyderabad, Telangana. In the present study, endo and exoglucanase production from <i>Humicola</i> sp. SKESMBKU03 was studied and optimization of the cultural conditions to enhance production of enzymes has been reported. Among the twenty fungal cellulase producers isolated from different thermogenic habitats, the fungal strain 'HD3' identified as <i>Humicola</i> sp. SKESMBKU03 exhibited highest cellulase activity by plate screening assay. To enhance the production level of the enzyme, different cultural conditions were optimized and observed that optimum pH and temperature for endo and exoglucanase production was 5.0 and 45°C respectively. Maximum growth as well as enzyme production was recorded on 3rd day of incubation period in shake flask (100RPM) containing Mandel's Weber medium. Urea and malt extract among the organic nitrogen sources while ammonium chloride as inorganic nitrogen source were found to be the best nitrogen source (0.2%) for endo and exoglucanase production. The endo and exoglucanase activities are higher in media containing glucose as their carbon source (1%) followed by xylose and lactose. The organism showed maximum dry weight in pH of 9.0-10.0, temperature of 45°C, cellulose as carbon sources, yeast extract and malt extract of nitrogen source. The endo and exocellulases produced by the <i>Humicola</i> sp. SKESMBKU03 are highly stable at pH 8.0 and temperature of 75°C. The results indicate that the endo and exocellulases produced by <i>Humicola</i> sp. SKESMBKU03 are more stable at high temperature and alkaline pH.</p>	<p>Article History: Received : 31-09-2014 Revised : 01-12-2014 Accepted : 12-12-2014</p> <p>Keywords: <i>Humicola</i> sp. Cellulases Optimization Characterization</p>
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INTRODUCTION

Thermophilic fungi are species that grow at a maximum temperature of 50°C or above, and a minimum of 20°C or above (Maheshwari *et al.*, 2000). Based on their habitat, thermophilic fungi have received significant attention in recent years as a source of new thermostable enzymes for use in production of ethanol (Olsson and Hahn, 1996), organic acids (Luo *et al.*, 1997), and other chemicals (Cao *et al.*, 1997). Cellulose is one of the main components of plant cell wall material and is the most abundant and renewable nonfossil carbon source on Earth. Degradation of cellulose to its constituent monosaccharides has attracted considerable attention for the production of food and biofuels (Sukumaran *et al.*, 2010). The degradation of cellulose to glucose is achieved by the cooperative action of endocellulases (EC 3.1.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and beta-glucosidases (EC 3.2.1.21). Endocellulases hydrolyze internal glycosidic linkages in a random fashion, which results in a rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. Exocellulases hydrolyze cellulose chains by removing mainly cellobiose either from the reducing or the non-reducing ends, which leads to a rapid release of reducing sugars but little change in polymer length. Endocellulases and exocellulases act synergistically on cellulose to produce

cellooligosaccharides and cellobiose, which are then cleaved by beta-glucosidase to glucose (Vlasenko *et al.*, 2010; Duo-Chuan *et al.*, 2011). An important impediment in the exploitation of cellulose is the fact that the production of cellulase is expensive, contributing as much as 50 % to the overall cost of hydrolysis. This is due to the low specific activity of cellulase, necessitating a large quantity of the enzyme for extensive hydrolysis. Considerable progress has been made in optimization of culture conditions (Yu *et al.*, 1998; Techapun *et al.*, 2002; Olsson *et al.*, 2003) and mode of cultivation (Xu *et al.*, 2002). The optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses (Xue-Cai Hao *et al.*, 2006). This work focus on factors relevant for the enhancement of enzymatic hydrolysis of cellulosic compounds using *Humicola* sp. SKESMBKU03 fungus as a potential producer of cellulases.

MATERIALS AND METHODS

Collection of Samples

Total 100 Samples from different thermogenic habitats (Zoo dump, Nests of birds, Industrial waste, Vegetable market compost, Mushroom compost, Horse dung manure, Decomposing litter, Soils from furnace area,

Cattle dung and Municipal waste) of Telangana (India) were collected. The samples were taken by means of sterilized spatulas and collected in sterile sealed polythene bags. The samples were then brought to the laboratory for microbiological study.

Isolation of Fungi

All the samples were processed within 1-2 hrs of collection. For isolation, dilution plate method was used (Apinis, 1963). Ten grams of sample was placed in 100 ml of sterile water and shaken for proper mixing and made in to dilution (10 fold), the sample of 1 ml of 10^{-4} , 10^{-5} dilution are placed in the Yeast extract starch agar medium (YpSs: yeast extract-5gm, starch-15gm, K_2HPO_4 -1gm, $MgSO_4$ -0.5gm per 1000ml of distilled water), Yeast-extract glucose agar (yeast extract-5gm, glucose -15gm, K_2HPO_4 -1gm, $MgSO_4$ -50gm per 1000ml of distilled water) medium (Cooney and Emerson, 1964). Streptomycin and rose bengal were added to the molten medium after autoclave, and the plates were incubated at 45°C for 3-4 days to identify the fungi. All the isolates from thermogenic habitats of Telangana were given a code from where it is isolated and identified based on their morphology and mycelial characters according to (Johri *et al.*, 1999; Salar *et al.*, 2007 and Jean *et al.*, 2000) and maintained on YpSs slants at low temperature (4°C) for further study.

Screening for Cellulase Enzyme

All the twenty isolates were screened for cellulolytic activity on selective carboxy methyl cellulose agar (containing grams/litre: $NaNO_3$ -2.0, KH_2PO_4 -1.0, $MgSO_4$. $7H_2O$ -0.5, KCl-0.5, carboxy methyl cellulose, sodium salt-2.0, peptone-0.2, and agar-17.0) medium (Sacin *et al.*, 2011). Plates were spot inoculated with spores of pure culture and incubated at 45°C. After 3 days of incubation plates were flooded with Gram's iodine (2.0g KI and 1.0g iodine in 300ml distilled water) for 5 minutes, the diameter of the zone of decolorization around each colony was measured (Ramesh *et al.*, 2008). The fungal colony showing largest zone of decolorization was selected for further studies on cellulase production.

Identification of Thermophilic Fungi

Isolate (HD3) showing large clear zone of cellulolysis on carboxy methyl cellulose agar is further identified to the species level by molecular analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). The rDNA was extracted from mycelium using the thermolysis method according to the protocols described by Zhang *et al.* (2010). The ITS regions were amplified by the polymerase chain reaction (PCR) with the universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') (Jingfeng *et al.*, 2013). The PCR products were purified and sequenced in both directions by Macrogen (Macrogen Inc., Geumchen-gu, South Korea). The sequences of fungus were compared using the GenBank database and basic local alignment search tools (Blast) for nucleotide analysis. The identification was confirmed with >98% similar to the fungal species deposited in GenBank. The sequence of the organisms is identified as *Humicola sp.* and deposited in EMBL and accession number is HG934775.1 (*Humicola sp.* SKESMBKU03).

Enzyme Production Medium

The four day old culture of *Humicola sp.* SKESMBKU03 was transferred to production medium

described by Mandel and Weber, (1969) containing milligrams/litre : $(NH_4)_2SO_4$ -1,400, KH_2PO_4 -2,000, $CaCl_2$. $2H_2O$ -300, $MgSO_4$. $7H_2O$ -300, $FeSO_4$. $7H_2O$ -5.0, $MnSO_4$. H_2O -1.6, $ZnSO_4$. $7H_2O$ -1.4, $CoCl_2$. $6H_2O$ -2.0, Peptone - 100, Tween-80 - 100, pH was adjusted to 5.5 and carbon sources were added at a concentration of 1 percent was used for all the fermentations. The inoculated medium was incubated at 45°C in shaker incubator for 3, 6, 9, 12 days. At the end of the fermentation period, the culture medium was filtered through Whatman No.1 filter paper to obtain the crude extract, which served as enzyme source.

Enzyme Assay

The carboxymethyl cellulose (CMCase) or endoglucanase activity determined according to the method by Ghose, (1987) reaction mixture contains 0.5ml of 1% carboxymethyl cellulose solution, 1ml of 0.05M phosphate buffer, (pH 5.5) and 0.5ml enzyme filtrate was incubated at 60°C for 30 minutes and releasing reducing sugars were estimated against blank. Exoglucanase activity was determined by using cellulose powder as a substrate. The reaction mixture (2 ml) contained 1 ml of 1% suspension of the substrate and 1ml of enzyme filtrate and incubated for 1 hour at 60°C, enzyme activity was expressed as International units (IU) (Bhat and Maheshwari, 1987). Absorbance of the above solution was measured at 575 nm, One international unit of cellulase activity is the amount of enzyme that forms 1 μ mol glucose (reducing sugar as glucose) per minute during the hydrolysis reaction. Reducing sugars were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959).

Effect of pH on Endo and Exoglucanses Production

The effect of pH on endo and exocellulase production was assessed by cultivating *Humicola sp.* SKESMBKU03 in 150ml flask containing 25ml of optimized media of varied pH ranging from 3.0-10.0, the pH of the medium was adjusted by using 1N HCl or 1N NaOH and incubated at 45°C in shaker incubator (100RPM) for 3, 6, 9 and 12 days at regular intervals the enzyme was extracted and used as endo and exocellulase source (Roberto *et al.*, 2005).

Effect of Temperature on Endo and Exoglucanses Production

In order to determine the optimum temperature for endo and exocellulase production by *Humicola sp.* SKESMBKU03, the fermentation was carried out at different temperatures ranging from 35, 45, 50 and 55 °C for 3, 6, 9, 12 days. Enzyme activity was estimated at each temperature and time period, according to standard assay procedure (Gomes *et al.*, 2000).

Optimization of Carbon and Nitrogen Sources on Endo and Exoglucanses Production

To identify a suitable carbon source for the endo and exoglucanses production by *Humicola sp.* SKESMBKU03, the various carbon sources were tested (maltose, fructose, glucose, lactose, xylose, starch, carboxy methyl cellulose, cellulose and sucrose). These carbon sources were tested individually at the concentration of 1% with production medium and incubated at 45°C in shaker incubator for 3, 6, 9 and 12 days. To investigate the effect of organic and inorganic nitrogen sources on the endo and exoglucanses production, experiments were carried out with addition of different organic and inorganic organic nitrogen sources

namely peptone, yeast extract, malt extract, beef extract, urea. $(\text{NH}_4)_2\text{SO}_4$, NaNO_3 , KNO_3 , NH_4Cl , KH_2PO_4 were added at a concentration of 0.2% to the Mandel and Weber production medium. All the flasks were incubated at $45^\circ\text{C} \pm 2$ in an orbital shaker incubator at 100 RPM. At regular interval enzyme assay was carried out (Coronei *et al.*, 1991).

Effect of Static and Agitated Condition on Cellulase Production

To study the effect of static and agitated condition on cellulase production by fungus, 2 sets of fermentation medium was prepared. In all the sets, all the conditions (pH, temperature) applied were kept constant. One set of inoculated medium was kept in an incubator without shaking while other sets were kept in different RPM (100,150,200) in an orbital- shaker incubator. Cellulase activity was determined by assaying culture filtrate on 3,6,9,12days of incubation (Shahriarinoor *et al.*,2011).

Stability Studies on Endo and Exogulcanses

The thermal stability was investigated by measuring the enzyme activity after keeping the aqueous enzyme solution for 1 hour at temperatures between 35°C and 80°C in the absence of substrate and at constant pH 5.5. Remaining enzyme activity was determined by enzyme assay. The pH stability (pH 3.0 to10.0) of the crude enzyme was evaluated by mixing the enzyme solution and buffer to give final proportion of 0.5:1 (v/v). These solutions were incubated at 45°C for 1 hour and remaining activity was determined by enzyme assay. (Quiroz-Castaneda *et al.*,2009)

Determination of Fungal Biomass

At regular intervals of time (3, 6,9 and 12 days) the contents of the flasks were aseptically passed through pre-weighed Whatman No.1 filter paper to separate mycelial mat from culture filtrates. The filter papers, along with mycelial mat were dried at 70°C in an oven for overnight and weight was recorded. The difference between the weight of the filter paper bearing mycelia mat and weight of pre-weighed filter paper represented fungal biomass, which was expressed in terms of dry weight of mycelia mat in milligrams (Shilpi *et al.*, 2011).

RESULTS

Collection of Samples and Isolation of Fungi

In total, of 100 samples, 20 isolates were selectively obtained from different thermogenic habitats of Telangana (India) based on the methods described in Materials and Methods. It has found that yeast extract starch agar medium found good for the growth of thermophilic fungi compare to yeast extract glucose agar medium.

Screening and Identification of Thermophilic Fungi for Cellulase Production

All the twenty isolates were screened for cellulolytic activity. All the tested strains were capable of producing cellulose, but in varying degrees (Table 1). The most potent species for cellulase production was *Humicola sp.* SKESMBKU03 isolated from horse dung manure with plate clearing zone of 3.5 cm in diameter (Figure 1 and 2). Which was morphologically (Colonies on YpSs agar at 45°C are white at first but soon turns through jet-black as spore maturation proceeds. Hyphae colourless, prostrate, branched, septate, 2-5 μm wide. Conidiogenous cells small, $8.7 \times 3.7 \mu\text{m}$. Conidia dark brown, smooth walled, translucent, generally globose, 7-12.5 μm in diameter, or

oval 11.2-14.6 \times 7.5-10 μm , produced on hyphal branches or developed intercalarily.) and molecularly identified and deposited in EMBL, accession number HG934775.1 (Figure 3). The pure culture of fungi was made by the hyphal tip method and maintained at 4°C for further use.



Figure 1: Culture plate and microscopic image of *Humicola sp.* SKESMBKU03

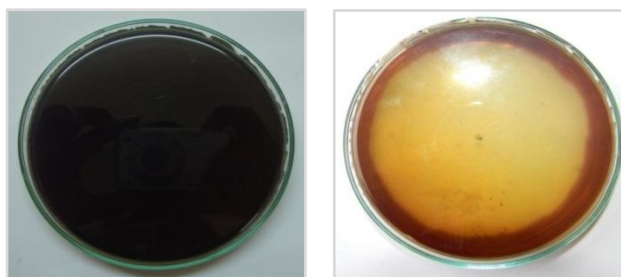


Figure 2: Control and Cellulolytic plate of *Humicola sp.* SKESMBKU03

Table 1: Twenty isolates of thermophilic fungi and zone of cellulolysis in Centimeters

No	Name of the Organism	Zone of Cellulolysis (in Cm)
1	HD1	2.5
2	HD2	2.0
3	HD3	3.5
4	CD1	2.0
5	CD2	2.5
6	MC1	1.5
7	MC2	1.5
8	CM1	2.5
9	CM2	3.0
10	MSW1	2.5
11	MSW2	2.5
12	MSW3	3.0
13	DC1	1.0
14	DC2	2.5
15	ZD1	2.0
16	ZD2	2.5
17	NM1	2.0
18	NM2	2.0
19	CMS1	1.5
20	CMS2	2.0

NOTE: HD = HORSE DUNG, CD = CATTLE DUNG, MC = MUSHROOM COMPOST CM = CHICKEN MANURE MSW= MUNICIPAL SOLID WASTE, DC = DECOMPOSING LITTER ZD = ZOO DUMP, NM = NEST MATERIAL BIRDS, CMS = COAL MINE SOIL (Various thermogenic habitats from where samples were collected).

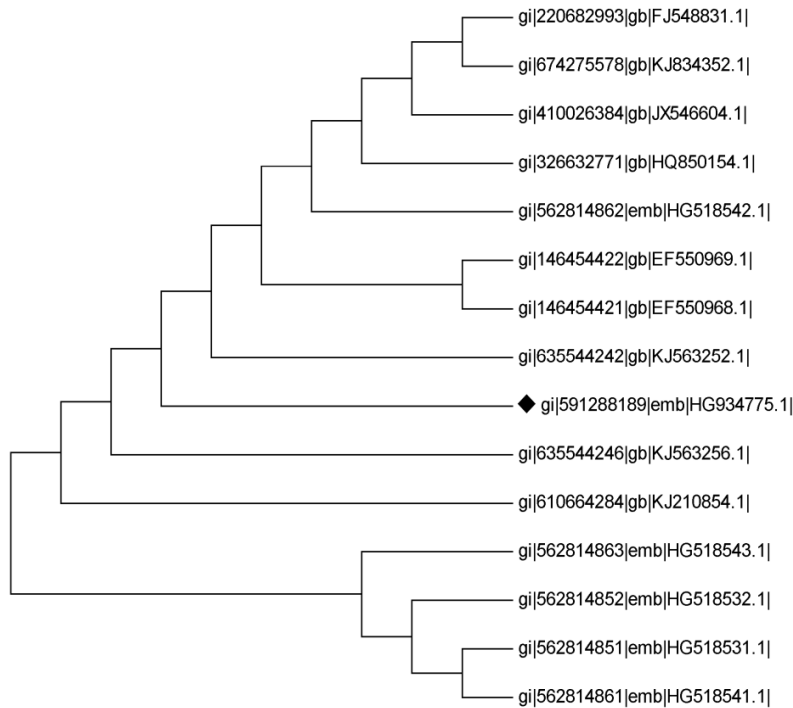


Figure 3: Phylogenetic tree was constructed using MEGA-5 (Molecular Evolutionary Genetics Analysis) software by the neighbor joining method

Effect of pH on Endo and Exogulcanses Production

Humicola sp. SKESMBKU03 shows the highest production of endo and exoglucanase at pH 5.0 and 6.0

on third day of incubation, as the incubation proceeds the endo and exoglucanase activity was decreased (Table 2).

Table 2: Effect of pH on endo and exogulcanses production

Name of thermophilic fungus	pH	Endoglucanase Activity/ U/ml ⁻¹				Exoglucanase Activity/ U/ml ⁻¹			
		Incubation period (days)				Incubation period (days)			
		3 rd	6 th	9 th	12 th	3 rd	6 th	9 th	12 th
<i>Humicola sp.</i> SKESMBKU03	pH-3	ND	ND	0.14	0.211	ND	ND	0.022	0.029
	pH-4	ND	0.035	0.088	0.096	ND	0.008	0.022	0.019
	pH-5	1.193	0.211	0.15	0.048	0.049	0.052	0.037	0.016
	pH-6	0.311	0.27	0.088	0.035	0.012	0.036	0.06	0.008
	pH-7	0.077	0.162	0.096	ND	ND	0.04	0.033	ND
	pH-8	0.112	0.04	0.035	0.011	0.022	0.025	0.027	0.025
	pH-9	0.176	0.109	ND	ND	ND	0.022	0.019	ND
	pH-10	0.088	ND	ND	ND	ND	ND	ND	ND

ND= No activity detected

Table 3: Effect of pH on dry weight of mycelium

Name of thermophilic fungus	pH	Dry weight of mycelium in milligrams (mgs)			
		Incubation period (days)			
		3rd day	6th day	9th day	12th day
<i>Humicola sp.</i> SKESMBKU03	pH-3	60	70	90	110
	pH-4	30	50	60	80
	pH-5	40	50	50	80
	pH-6	40	50	60	80
	pH-7	40	60	90	120
	pH-8	60	70	100	120
	pH-9	60	80	120	160
	pH-10	40	70	120	160

Effect of Temperature on Endo and Exogulcanses Production

The effect of temperature on the endo and exogulcanses production was studied at the temperature

range from 35-55 °C (Table 4).The results of the test showed that the optimal temperature for endo and exocellulases was 45 °C. Meager amount of enzyme produced at 50 °C on 3rd day of incubation.

Table 4: Effect of temperature on endo and exogulcanases production

Name of thermophilic fungus	Temperature in °C	Endoglucanase activity/ U/ml ⁻¹				Exoglucanase activity/ U/ml ⁻¹			
		Incubation period (days)				Incubation period (days)			
		3 rd day	6 th day	9 th day	12 th day	3 rd day	6 th day	9 th day	12 th day
<i>Humicola sp.</i> SKESMBKU03	35°C	0.118	ND	ND	ND	ND	ND	0.079	ND
	45°C	1.19	0.576	0.34	0.3	0.298	0.205	0.106	0.075
	50°C	0.098	0.05	ND	ND	0.083	0.052	0.03	ND
	55°C	0.133	0.07	ND	ND	0.037	0.024	ND	ND

ND= No activity detected

Table 5: Effect of temperature on dry weight of mycelium

Name of thermophilic fungus	Temperature in °C	Dry weight of mycelium in milligrams (mgs)			
		Incubation period (days)			
		3 rd day	6 th day	9 th day	12 th day
<i>Humicola sp.</i> SKESMBKU03	35°C	50	70	80	100
	45°C	20	70	120	160
	50°C	50	60	80	100
	55°C	50	70	80	100

Optimization of Carbon and Nitrogen Sources on Endo and Exogulcanases Production

To evaluate the carbohydrates to cause induction or repression of cellulase, the organism was grown on different carbon sources. In general, enhanced production

of enzyme was observed with all the tested sugars. Among the carbon sources examined, glucose found to be the best inducer in SmF on third day of incubation next to this is xylose (Table 6).

Table 6: Optimization of carbon sources on endo and exogulcanases production and dry weight of mycelium

Name of thermophilic fungus	Carbon sources	Endoglucanase activity/ U/ml ⁻¹				Exoglucanase activity/ U/ml ⁻¹			
		Incubation period (days)				Incubation period (days)			
		3 rd day	6 th day	9 th day	12 th day	3 rd day	6 th day	9 th day	12 th day
<i>Humicola sp.</i> SKESMBKU03	Glucose	1.198	0.576	0.34	0.3	0.298	0.205	0.106	0.075
	Xylose	0.769	0.84	0.3	0.211	0.045	0.055	0.037	0.012
	Cellulose	0.011	0.048	0.011	0.003	0.03	0.022	0.019	0.007
	Starch	0.003	0.023	0.025	0.05	0.007	ND	ND	ND
	CMC	0.007	0.008	0.148	0.045	0.002	0.012	0.015	0.036
	Sucrose	0.007	0.04	0.038	0.025	0.005	0.014	0.002	0.002
	Maltose	0.203	0.059	0.048	0.029	0.007	0.014	0.118	0.042
	Fructose	0.15	0.192	0.318	0.077	0.079	0.077	0.077	0.043
	Lactose	0.011	0.029	0.14	0.077	0.178	0.174	0.022	0.014

ND= No activity detected

Table 7: Effect of carbon sources on dry weight of mycelium

Name of thermophilic fungus	Carbon sources	Dry weight of mycelium in milligrams (mgs)			
		Incubation period (days)			
		3 rd day	6 th day	9 th day	12 th day
<i>Humicola sp.</i> SKESMBKU03	Glucose	30	70	80	100
	Xylose	40	60	80	90
	Cellulose	20	60	130	150
	Starch	30	60	80	100
	CMC	20	30	40	80
	Sucrose	70	80	90	100
	Maltose	70	90	90	100
	Fructose	70	80	100	130
	Lactose	70	90	100	120

Effect of different organic as well as inorganic nitrogen sources on cellulase production by *Humicola sp.* SKESMBKU03 were studied. Among the various nitrogen

sources tested urea and malt extract were found to be the most effective for production of endo and exo glucanase activity (Table 8).

Table 8: Effect of nitrogen sources on endo and exogulcanses

Name of Thermophilic Fungus	Nitrogen sources	Endoglucanase Activity/ U/ml ⁻¹				Exoglucanase Activity/ U/ml ⁻¹			
		Incubation Period (days)				Incubation Period (days)			
		3 rd day	6 th day	9 th day	12 th day	3 rd day	6 th day	9 th day	12 th day
<i>Humicola sp.</i> SKESMBKU03	Peptone	0.214	0.155	0.144	ND	0.030	0.036	0.037	0.029
	Yeast extract	0.059	0.029	0.007	0.003	0.022	0.062	0.063	0.008
	Malt extract	0.144	0.140	ND	ND	0.093	0.04	0.04	0.007
	Beef extract	0.011	0.133	0.118	ND	0.007	0.027	0.029	ND
	Urea	0.484	0.192	0.14	0.011	0.002	0.044	0.052	0.073
	(NH ₄) ₂ SO ₄	0.214	0.029	ND	ND	0.053	ND	ND	ND
	NaNO ₃	0.227	0.233	0.233	0.273	0.009	0.011	0.028	0.06
	KNO ₃	0.029	0.227	0.233	0.294	0.044	0.038	0.038	ND
	NH ₄ Cl	0.233	0.412	0.24	0.262	0.053	0.007	0.007	ND

ND= No activity detected

Table 9: Effect of nitrogen sources on dry weight of mycelium

Name of thermophilic fungus	Nitrogen sources	Dry weight of mycelium in milligrams (mgs)			
		Incubation period (days)			
		3 rd day	6 th day	9 th day	12 th day
<i>Humicola sp.</i> SKESMBKU03	Peptone	100	110	130	160
	Yeast extract	120	130	150	160
	Malt extract	100	120	140	160
	Beef extract	60	80	90	110
	Urea	30	40	60	100
	(NH ₄) ₂ SO ₄	60	80	90	110
	NaNO ₃	50	70	90	110
	KNO ₃	70	80	90	100
	NH ₄ Cl	90	100	120	130

Effect of Static and Agitation Condition on Endo and Exogulcanses Production

When cellulase production was studied under static and shake flask conditions (100, 150 and 200 RPM) for 3, 6, 9 and 12 days at 45 °C. The enzyme production by

Humicola sp. SKESMBKU03 was found to be high in agitation condition in comparison with that of static conditions. It was noticed that the optimum level of rotation needed for the maximum production for both endo and exogulcanses was at 100 RPM (Table 10).

Table 10: Effect of static and agitation condition on endo and exogulcanses production

Name of Thermophilic Fungus	RPM and Static condition	Endoglucanase activity/ U/ml ⁻¹				Exoglucanase activity/ U/ml ⁻¹			
		Incubation period (days)				Incubation period (days)			
		3 rd	6 th	9 th	12 th	3 rd	6 th	9 th	12 th
<i>Humicola sp.</i> SKESMBKU03	100	1.198	0.576	0.34	0.3	0.298	0.205	0.106	0.075
	150	ND	ND	ND	ND	ND	ND	ND	ND
	200	ND	ND	ND	ND	ND	ND	ND	ND
	Static condition	0.37	ND	ND	ND	0.184	0.092	ND	ND

ND= No activity detected and RPM= Rotations per minute

Table 11: Effect of static and agitation condition on dry weight of mycelium

Name of Thermophilic fungus	RPM and Static condition	Dry weight of mycelium in milligrams (mgs)			
		Incubation period (days)			
		3 rd	6 th	9 th	12 th
<i>Humicola sp.</i> SKESMBKU03	100	50	80	110	1120
	150	20	60	80	1110
	200	50	60	80	1100
	Static condition	60	80	90	1100

RPM= Rotations per minute

Stability Studies on Endo and Exogulcanses

The effect of pH on endo and exogulcanses were shown in (Figure 4). Samples of the enzyme solutions in buffer of various pHs were incubated at 45°C for 1hr and then the remaining activities were assayed. The activities are stable between pH 5.0 and 7.0 for endoglucanase, between pH 5.0 and 6.0 for exoglucanase. Enzyme activity was decreased with enhanced pH treatment. Activity assay at different temperatures (35-85°C) showed by endo and

exogulcanses had activity over a broad range of temperatures with maximum at 45-75°C for both the enzymes (Figure 5).

Determination of Fungal Biomass

The biomass production of *Humicola sp.* SKESMBKU03 denotes that biomass increased with incubation period. The results revealed that the *Humicola sp.* SKESMBKU03 showed increased biomass in pH of

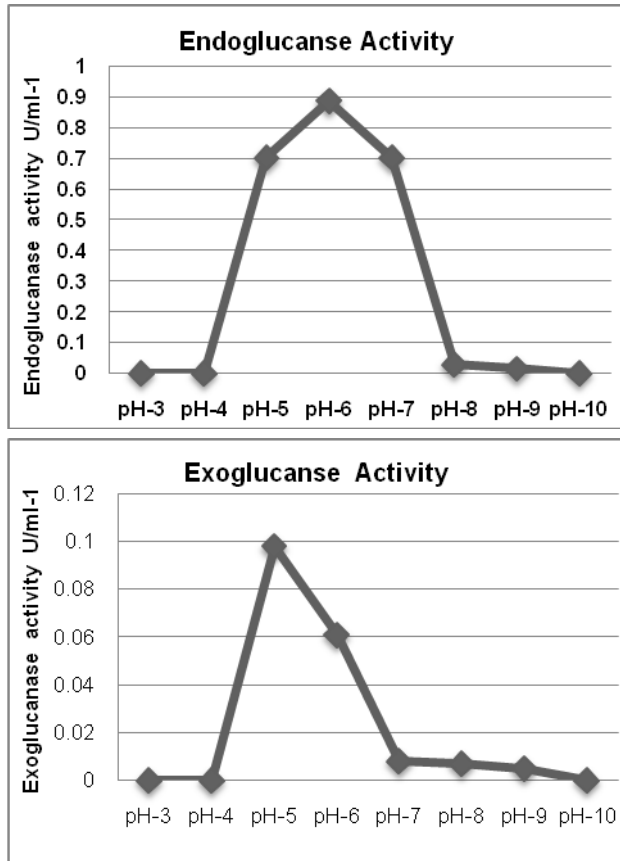


Figure 4: Stability of enzyme at different pH

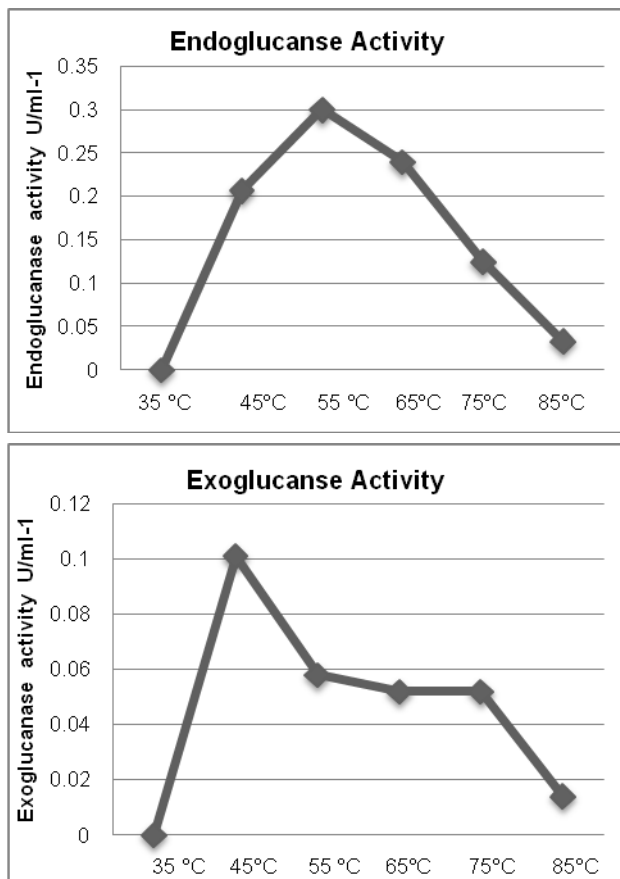


Figure 5: Stability of enzyme at different temperatures

5.0 – 6.0 (Table 3) and temperature of 45°C (Table 5). Cellulose found to be best carbon sources for biomass production followed by fructose and lactose (Table 7). In nitrogen sources peptone, yeast extract and matl extract were found to produce maximum fungal biomass (Table 9). Compare to the static, maximum fungal biomass was produced in the shake flask culture at 100RPM (Table 11). There is no co-relationation between endo and exoglucanases production and biomass production.

DISCUSSION

The conversion of cellulosic biomass by microorganisms is a potential sustainable approach to develop novel bioprocesses and products. Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications (Henrissat *et al.*, 1998; Ramesh Chander Kuhad *et al.*, 2011). Cellulase research has been concentrated mostly in mesophilic fungi but there is increasing interest in cellulase production by thermophilic fungi due to their higher growth rate and thermostable and alkali stable properties. The development of rapid and reliable methods for the screening of cellulases from thermogenic inhospitable environments will allow a greater number of novel fungal cellulases to be isolated with purpose of industrial use.

The aim of the present work was to isolate and identify a high cellulase producing thermophilic fungus from thermogenic habitats and optimization of media components. A number of samples were processed for isolation of thermophilic fungi which were later screened for the cellulase production. Pure fungal organisms from thermogenic habitats were screened on CMC agar plates supplemented with Grams iodine. Zone of clearance was observed for all the fungal isolates in varying degree. Among the isolates *Humicola sp.* SKESMBKU03 produced a zone of 3.5 cm in diameter. Based on the colony morphology and microscopic observation, it was identified as *Humicola sp.* SKESMBKU03.

Transportation of various chemical across the cell membrane, including movement of enzyme and their activity is importantly influenced by the pH of the medium. There are general reports showing that different nitrogen sources have different influences on extracellular enzyme production by different strains. Current finding also shows that pH of production medium was an important factor affecting endo and exoglucanase production (Table 2). Due to the change in the pH value from slightly acidic (pH-5.5) to more acidic (pH 2-3) condition which is unfavorable for the production of endo and exoglucanase activity. The effect of pH on endo and exoglucanase production by this fungi supports the findings of Moretti *et al.*, (2012) who has reported the optimum pH for endo-glucanases and xylanase from *Aspergillus fumigatus* M.7.1 were 4.5 and 4.5-5.5, respectively and 5.0 for both enzymes from *Myceliophthora thermophila* M.7.7.

In Table 4 results shows that the enzyme activity was decreased when the temperature increased above 50°C. The investigations are in harmony with the findings of Irshad *et al.*, (2011) who found that xylanase production by *H. lanuginosus* is high at 45°C and low at 50°C.

Most fungi can selectively use substrates from a mixture of different carbon sources. The presence of preferred carbon sources effect its activity. Endo and

exoglucanase production was found to be influenced by the nature of the carbon source used in the production media. Glucose was found to be good enhancer for endo and exoglucanase production by *Humicola sp.* SKESMBKU03. The results are nearer to the findings of Mchunu *et al.* (2013) who found that *Thermomyces lanuginosus* utilized xylose, trehalose, raffinose, D-mannose, furanose, fructose and glucose as best carbon source.

Cellulase production was dependent on the nature of nitrogen source in the culture medium. Various inorganic and organic nitrogen sources were tested for their effect on cellulase production. The maximum enzyme activities were present in organic nitrogen sources urea (endoglucanase), malt extract (exo-glucanase). The results are in agreement with Coutts and Smith, (1976) who found that urea and NaNO₃ seemed to be most suitable for Cx and C1 cellulase production by *Sporotrichum thermophile*.

It was noticed that the optimum level of rotation needed for the maximum production of enzyme. The maximum production of both exo-endoglucanase is achieved at 100RPM. Further increase in RPM level, there was decrease in enzyme activity, this could be due to the fact that the increase in RPM level has resulted in the coagulation of the organism to form clumps and decrease in enzyme production. Tarek *et al.* (2013) who establish that rate of cellulase production was five times more in shaking cultures than in static ones, while β -glucosidase was seven times more in shaking cultures than in static ones in *Sclerotium rolfsii*.

Humicola sp. SKESMBKU03 was isolated from semi-arid regions of Telangana and grows better at high temperature, indicating that the optimal growing temperature does not necessarily correlate with the optimal condition for cellulases activity. pH Stability of exo-endoglucanase was found to be 5.0-7.0 and 5.0-6.0 respectively. Though cellulase activity decreased at elevated temperatures still substantial activity was maintained. Thermostability profile of cellulase showed that enzyme was thoroughly stable at 50-60°C for 1hr. The results are in consensus with findings of Busk and Lange, (2013) who reported that with increase in temperature there is a decrease in the stability of cellulase activity by *Talaromyces leycettanus*.

Determination of fungal biomass was done by taking dryweight of mycelium and it was found that there is a difference in biomass production in various media components. The maximum biomass was produced in pH of 9-10, temperature of 45°C. Production media containing cellulose, peptone and yeast extract as their carbon and nitrogen sources showed a good biomass production. The findings are in scenario with the investigations of Granjo *et al.*, (2007) who found that the biomass will be less in the beginning of the incubation periods later on increase in the incubation period there will be increase in biomass production.

CONCLUSIONS

The result has enabled the ideal formulation of media composition for maximum endo and exoglucanase production by *Humicola sp.* SKESMBKU03. The high activity and stability of cellulase enzymes between neutral to alkaline pH and high temperature will be of use in various industrial and biotechnological applications.

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