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Purification and characterization of an extracellular cellulase produced using alkali pretreated rice straw by *stenotrophomonas maltophilia*

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Abstract
Cellulase producing bacteria were isolated from biogas slurry samples. Out of 24 isolates, JSR-001, identified as *Stenotrophomonas maltophilia* through 16S rRNA based molecular phylogenetic approach, showed maximum cellulase activity and it was selected for further enzyme production. Enzyme production was carried out using cheap and easily available agro-waste; AFEX-pretreated, rice straw as a growth supporting material. The cellulase enzyme was extracted and purified by Ammonium Sulfate precipitation (80%), dialysis (5kDa tubing cellulose membrane) followed by ion-exchange chromatography (Q-sepharose) and gel filtration chromatography using Sephadex G-200. The SDS-PAGE and zymogram analysis revealed the molecular weight of the produced cellulase enzyme to be ~38kDa. The optimum temperature and pH for enzyme activity were found to be 50ºC and 8.0 respectively. This purified cellulase enzyme having Km of 5.41mg/ml and a Vmax of 161.29 µmol/min/mg was found to be functionally stable even in the presence of 15% Sodium Chloride. It was also unaltered by ethanol, methanol, and isopropanol but partially inhibited by toluene and acetonitrile. The same was strongly activated by additives and detergents like Co^{2+}, Na^{+}, K^{+}, Zn^{2+}, SDS, Triton X-100 and Tween-20. Lignocellulosic substrates were also effectively degraded by this enzyme. Since the purified cellulase enzyme produced using our cheap substrate is halo and thermostable and also capable of degrading cellulosic substrates, it is apparent that it could be potentially considered in detergent, pulp and paper industries.

Keywords: cellulase, substrate pretreatment, halo tolerant, alkali tolerant, biogas slurry, rice straw

1. Introduction
Lignocellulosic biomass is the most available and renewable energy source in plenty. It is made up of cellulose, hemicelluloses, and lignin, which are bound together to form a complex structure. Annually, plants produce about 4 x 10^9 tons of cellulose along with other polysaccharides (Yin *et al.*, 2010) [1]. Accumulation of large volumes of lignocellulosic waste material leads to serious environmental problems (Vivek *et al.*, 2008) [2]. Conversion of cellulosic material to simple sugars by microorganisms plays a critical role in the carbon cycle (Romano *et al.*, 2013) [3]. Cellulose comprises long polymers of β 1-4, linked glucose molecules. It had already been reported that a set of cellulases including endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrlase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) is required for complete hydrolysis of cellulosic materials (Perez *et al.*, 2002) [4]. Cellulase has a wide variety of industrial applications including leather, textile, agriculture, food, paper, pulp, animal feed industry, in waste/water management, in brewing and winemaking, in household laundry detergents and in bio-ethanol production (Bhat, 2000; Kim *et al.*, 2005; Begiun & Aubert 1994; Liu *et al.*, 2004; Cavaco-Paulo 1998; Ghose and Singh, 1993; Gupta *et al.*, 2011; Sukumaran *et al.*, 2009) [5-12]. At commercial scale, the majority of cellulase is synthesized using fungal species like *Aspergillus* and *Trichoderma* because of its high enzyme activity (Nandakumar *et al.*, 1994). [13] Meanwhile, bacterial genera were also reported for cellulase production, including *Bacillus*, *Cellulomonas*, *Clostridium* etc., (Roboson & Chambliss 1989) [14]. Due to stability at high temperature, short generation time and easier for the genetic purpose, bacterial cellulase is most commonly preferred (Nagendran *et al.*, 2009) [15].

Since plants are the major source for the substrates for cellulase production, many researchers have used cheap agricultural residues like sugarcane bagasse, wheat bran, rice bran and rice straw as a substrate for its production (Techapun *et al.*, 2003) [16]. However, because of the complex structure and rigid nature of plant cell walls, lignocellulosic plant waste material must be pretreated before enzymatic hydrolysis. Commonly used pre-treatment methods are alkaline, acid and steam explosion (Cabrera *et al.*, Zu *et al.*, & Liu *et al.*, 2014) [17-19]. According to previous reports by Sun and Chang (Sun *et al.*, 2002 & Chang *et al.*, 2000) [20, 21], alkali pre-treatment results in sugar degradation than acid pre-treatment comparatively and hence most commonly preferred.

In this study, the Ammonia Fiber Explosion (AFEX) pretreated (Santhi *et al.*, 2014) [22] rice straw was used as a substrate material for cellulase production using *Stenotrophomonas maltophila* JSR-001 isolated from the biogas plant slurry sample. Our report suggested that the activity and stability of cellulase enzyme from *S. maltophilia* have significant sustainability at alkaline pH, high temperature, and high saline conditions with potential industrial applications.

2. Materials and Methods
2.1 Microorganisms
Cellulolytic bacterial strain *Stenotrophomonas maltophilia* JS-

R001, isolated from a biogas slurry sample and identified through 16S RNA typing was used for this study. The strain was screened for cellulase production by using modified cellulose agar with the composition and culture conditions mentioned elsewhere (Santhi *et al.*, 2014) [23]. After incubation, Cellulose-agar plates were flooded with 0.1% Congo red (Teather and wood 1982) [24] and de-stained with 1M NaCl for 15mins. Then the plates were observed for the clearance zone around the colonies indicating cellulose degradation. Positive colonies were chosen and used for further studies. 40% Glycerol was used to stock the bacterial culture and stored at -20^{0}C. The culture was revived periodically and checked for the stability of cellulose degrading potential.

2.2 Production of Crude Cellulase

The cellulolytic bacterial strain was inoculated into Cellulase Production Media (CPM) as described elsewhere (Lo *et al.*, 2009) [25]. CPM has been supplemented with 1% of AFEX (Ammonia Fiber Explosion method) pretreated rice straw as a carbon source at pH 7.0. Here the 10g of chopped biomasses were exposed to liquid ammonia at 100 °C for a period of one hour. This treated bio-waste substrates were then sun-dried individually to reduce the moisture content and powdered (Santhi *et al.*, 2014) [22]. The isolate *S. maltophilia* was inoculated into the production medium and incubated at 37ºC with 180 revolutions per minute (RPM) for 72 hours. After the incubation period, the production media was centrifuged at 11290g for 30mins at 4ºC and the collected supernatant (Culture filtrate) was considered as the crude enzyme.

2.3 Enzyme activity assay

Cellulase activity was determined by following Teather and Wood (1982) [24] method using Carboxymethyl cellulose (CMC) as a substrate. The reaction mixture consisted of 0.5ml of diluted enzyme and 0.5ml of 1% (w/v) of CMC in 50mM Sodium Citrate buffer (pH-4.8) was incubated at 50ºC for 30min. The amount of reducing sugars released during the reaction was measured at 540nm by using the DNS method (Miller, 1959) [26] and D-glucose was used as a standard. One International Unit (IU) of cellulase activity is the amount of enzyme that forms 1µmol glucose (reducing sugars as glucose) per minute during hydrolysis reaction.

2.4 Determination of Protein Concentration

Protein content in the culture supernatant was estimated by Bradford (1976) [27] method with Bovine Serum Albumin (BSA) as standard. Following 5min of incubation at room temperature absorbance was measured at 595nm. All the experiments were performed in triplicates.

2.5 Purification of Cellulase

All purification steps were performed at 4ºC unless stated otherwise. *S. maltophilia* was grown for 72 hours and the crude supernatant was collected by centrifuging the broth at 11290g for 30mins. The crude supernatant was then subjected to 20-80% of Ammonium sulfate precipitation. The precipitate was again centrifuged at 11290×g for 30mins and the resulting pellet was reconstituted with 50mM Phosphate buffer (pH-8) and dialyzed against the same buffer for 48 hours. Dialysis was performed using a tubing cellulose membrane with a molecular cutoff of 5kDa (Sigma). This dialyzed sample was then subjected to ion-exchange chromatography on Q-Sepharose column and equilibrated with same 50mM Phosphate buffer. Fractions were collected with linear gradient 0f 0.1-1M NaCl in same equilibration buffer at a flow rate of 0.2ml/min. Each fraction was analyzed for cellulase activity and active fractions were pooled for further purification by gel filtration chromatography on Sephadex G-200 column. The enzyme was eluted using 50mM Phosphate buffer (pH-8) at a flow rate of 0.5ml/min and each fraction was assayed for cellulase activity. Fractions showing greatest activity were analyzed for purity and other characterization studies.

2.6 SDS-PAGE and Zymogram analysis

The purified enzyme was subjected to Sodium Dodecyl Sulfate –Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the method of Laemmli (1970)[28]. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. For reference molecular weight, the standard protein ladder was used (Fermentas). Zymography was performed on Native PAGE by incorporating the 0.1% of CMC in the separating gel according to the modified method of Schwartz *et al.*, (1987) [29]. Electrophoresis was performed at 4ºC. Native gel was then soaked in 50mM phosphate buffer (pH-8) for 1 hour at 4ºC prior to staining with 0.5% Congo red. The gel was destained with 1M NaCl at 4ºC for overnight.

2.7 Effect of pH on Enzyme Activity and Stability

Effect of pH on enzyme activity was studied at pH values ranging from 3-11. The pH was adjusted using different buffers namely; pH 3.0-6.0 (0.05M citrate buffer), pH 6.5-8.0 (0.05M sodium phosphate buffer), pH 8.5-9.0 (0.05M Tris-HCl), pH 9.5-11 (0.05M glycine-NaOH) with 1% CMC as substrate. The reaction mixture with these buffers was incubated at 50ºC for 30 mins. The cellulase activity was measured by DNS (Dinitro Salicylic Acid) method. Effect of pH on enzyme stability was measured by pre-incubating the enzyme with different pH buffers at room temperature for 24 hours. The residual enzyme activity was recorded (DNS method) at every 4-hour intervals during the 24 hours of incubation.

2.8 Effect of Temperature on Enzyme Activity and Stability

The optimum temperature for high enzyme activity was determined by incubating the purified enzyme with 1% CMC in 50mM phosphate buffer (pH-8) at different temperatures between 20-100ºC for 24 hours. Standard assay (DNS method) conditions were performed for calculating enzyme activity. The residual enzyme activity was recorded at every 4-hour intervals during the 24 hours of incubation to evaluate the effect of temperature on its stability.

2.9 Effect of Salt Concentration on Enzyme Activity and Stability

The effect of salinity on enzyme activity was analyzed by pre-incubating the enzyme with 1% CMC, with different concentration of NaCl (5-30%) at specified assay conditions.

DNS method was used for calculating cellulase activity. To determine the stability, the enzyme was incubated with a salinity range of 5-30% for 24 hours at room temperature. The residual enzyme activity was recorded at every 4 hour intervals during the 24 hours of incubation.

The enzyme activity was measured using the enzyme activity assay, as described above. The values were expressed in percentages of the observed enzyme activity. Each value is an average of triplicate tests.

2.10 Effect of Metal Ions and Solvents on Enzyme Activity

The effect of various metal ions on enzyme activity was determined by incubating the enzyme with following metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Zn^{2+}, and Co^{2+}) for 1 hour at room temperature in 50mM phosphate buffer (pH-8). The activity was measured by the DNS method as described earlier.

The effect of solvents namely ethanol, methanol, butanol, toluene, DMSO, benzene, isopropanol, Glycerol, Acetonitrile and acetone on the enzyme activity was assessed. The 5% (v/v) of each solvent were added to reaction mixtures in a separate set of experiments and the residual enzyme activity was measured by DNS method.

A control reaction was carried out without metal ion & solvents and it was recorded as 100%. Each value is an average of triplicate tests.

2.11 Effect of Inhibitor, Chelator, and Denaturants on Enzyme Activity

The effect of inhibitor, chelating agent and denaturant were studied on the activity of the purified enzyme with two different concentrations. Cysteine inhibitor (β-mercaptoethanol), the chelator of divalent cation (EDTA) and denaturant (Urea) were studied at 1mM and 10mM in the reaction. The assay was carried out under standard assay conditions as mentioned previously.

2.12 Effect of Detergents on Enzyme Activity

Effect of detergents such as SDS, Triton X-100 and Tween-20 on the activity of cellulase produced were studied at concentrations of 0.1% and 1% and pre-incubated with the enzyme at 50ºC for 30mins. Assays were performed under standard assay conditions.

2.13 Enzyme Kinetic Studies

Kinetic studies were performed with different concentrations of CMC (1-10mg/ml) in 50mM phosphate buffer (pH 8) at 50ºC for 30mins. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined from a Lineweaver- Burk plot.

2.14 Degradation of lignocellulosic biomass

Lignocellulosic substrates like rice straw and sorghum stubbles were treated with the purified enzyme from *S. maltophilia* at a concentration of 29.37U/g at 50ºC for 2 hours (pH-8). Degradation of lignocellulosic biomass by cellulase from *S. maltophilia* was examined by Scanning Electron Microscopy (SEM) as previously reported by Li *et al.,* (2010) [30]. Biomass treated with buffer was taken as a control.

3. Results and Discussion

3.1 Microorganism Characterisation

The lignocellulolytic bacterial isolates from biogas slurry were isolated by serial dilution and spread plate on CMC media plates. Totally 24 isolates (JS-R001- JS-R024) were isolated from slurry samples. Screening of cellulolytic bacteria was conducted by using the congo red test as a preliminary study for identifying cellulase producers. Among them, JS-R001 showed strong cellulose degrading activity and biggest clearance zone (4.0cm diameter) on the CMC agar plate and hence, it was selected for further studies. Based on the morphological, biochemical and 16S rRNA sequences, the bacterial strain was identified as *Stenotrophomonas maltophilia JS-R001*, rod-shaped, Gram-negative bacteria with 99% homology. Similar finding was previously reported by Samira *et al.,* (2011) [31] in which, *S.maltophilia* on the CMC agar plate showed the clearance zone diameter of 4.5cm. Several strains of *S.maltophilia* have been reported already for applications of alkaline protease production (Juhasz *et al.*, 2000) [32], applications involving bioremediation of polycyclic aromatic hydrocarbons (Kuddus and Ramteke 2011) [33] and thermostable xylanase production (Raj *et al.*, 2013) [34].

3.2 Enzyme Production and Purification

Cellulase enzyme was produced by *S.maltophilia* with optimum concentration and activity, when grown in the production medium supplemented with 1% of AFEX pretreated rice straw through the submerged fermentation process. Previous reports of Iqbal *et al.,* (2011) & Ojuma *et al.,* (2003) [35, 36] also used pre-treated agro-industrial waste products such as wheat straw, sawdust, bagasse and corncob as growth supported substrate for cellulase production. The cell-free supernatant was used as a crude enzyme and it was precipitated using Ammonium sulfate by 20-80% saturation. Out of this, precipitated enzyme from 80% of saturation (Fig.1) was chosen for further studies which showed maximum specific activity (4.21 U/mg). This result was found to be in coherence with the previously reported studies on cellulase production (Sharma & Chand., 2012; Rawat & Tewari., 2012 and Ahmed *et al.,* 2015) [37-39]. The precipitate was dissolved in 50mM phosphate buffer (pH-8) and dialyzed against the same buffer. The dialyzed enzyme solution was subjected to ion-exchange chromatography in Q-Sepharose column and eluted with 1M NaCl in the same buffer. All the fractions were assayed for cellulase activity and fractions showing highest activity was pooled and stored at -20º C till further use (Fig. 2).

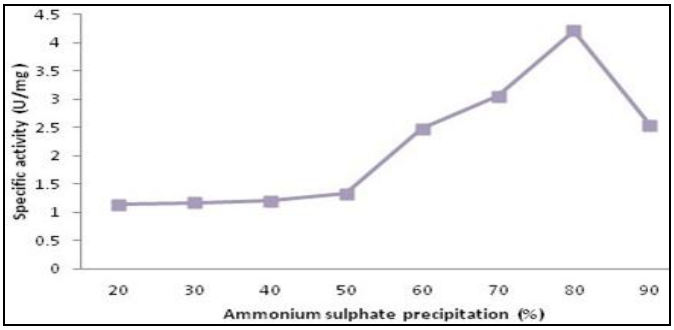


Fig 1: Precipitation of enzyme by Ammonium Sulfate precipitation method. The higher activity enzyme was obtained at 80% Ammonium Sulfate.

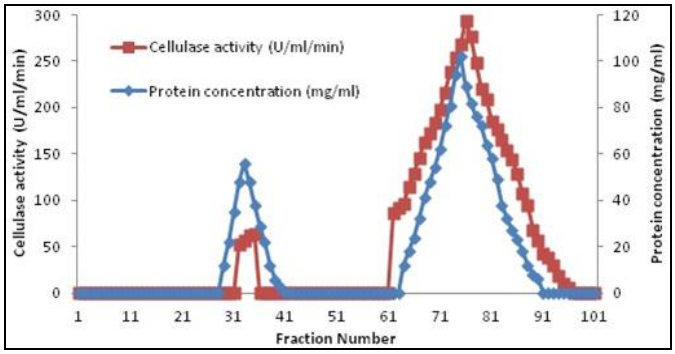


Fig 2: Active fractions from Anion exchange Q-chromatography. Fractions were subjected to total protein quantification and estimation of cellulase activity. Fraction no.32 and 80 showed higher protein concentration and enzyme activity.

The active fractions were further purified by Sephadex G-200 (Gel elution chromatography) and eluted with 0.1-0.5M NaCl. The enzyme was purified about 3.14 folds than crude enzyme with a recovery of 35.73%. It has the specific activity of 37.48U/mg. The summary of the purification is given in Table -1. Interestingly our method yielded highly active cellulase enzyme while comparing with the previous reports such as a study by Wang *et al.*, (2009) [40] who produced cellulase enzyme with a specific activity of 32.4U/mg and a recovery of 18.9% from *Salinivibrio sp*., using Q-sepharose and Sephadex G-200.

Table 1: Purification summary of Cellulase from *S.maltophilia.*

Purification Steps	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	262.26	105.2	2.49	1	100
$(NH_4)_2SO_4$ precipitation (80%)	204.3	48.5	4.21	2.15	77.89
Dialysis	167.1	14	11.93	2.83	63.72
Q -Sepharose	93.71	2.5	37.48	3.14	35.73
Sephadex G-200	59.8	1.2	49.83	20.01	22.8

3.3 SDS PAGE and Zymogram analysis

The purified cellulase was subjected to SDS-PAGE and activity was analyzed by Zymogram. The molecular mass of the purified cellulase from *S.maltophilia* JSR-001was found to be 38kDa (Fig-3).

In Zymogram also the cellulase enzyme exhibited a significant activity band corresponding to 38kDa. This observation is in coherence with previous reports of Ravi Kumar *et al.*, (2004) [41] (*Trichoderma reesei* cef 19 – 38kDa), Ganju *et al.*, (1990) [42] (*Chaetomium thermophile*-38kDa), Harsavardhan *et al.*, (2013) [43] (*Bacillus sp.*H1666 – 40kDa), and Potprommanee *et al.*, (2017) [44], (*Geobacillus sp.* HTA426 - 40kDa) (Fig-4).

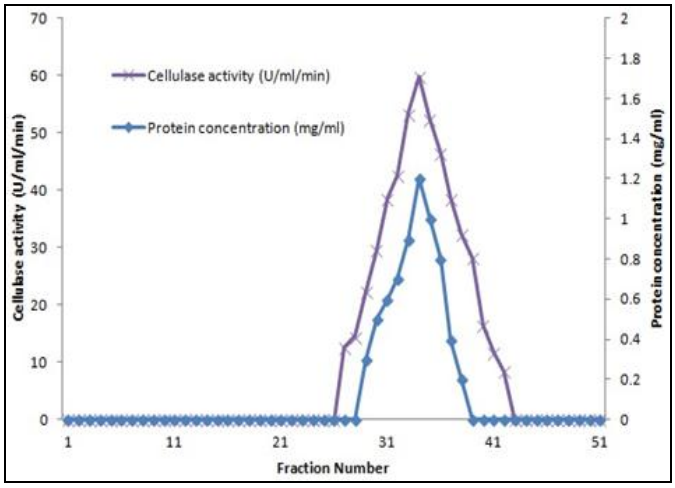


Fig 3: Active fractions from Gel Filtration Chromatography. Fractions were subjected to total protein quantification and estimation of cellulase activity. Fraction no.35 showed higher protein concentration and enzyme activity.

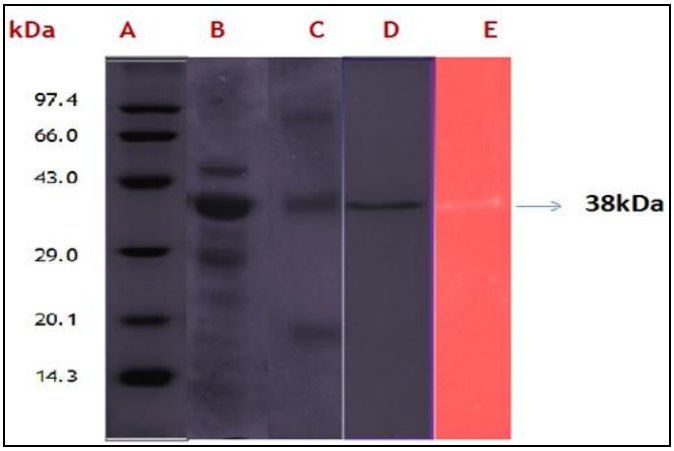


Fig 4: SDS-PAGE analysis of extracted cellulase produced by *S.maltophilia*. Lane A Protein marker, Lane B Dialyzed sample after Ammonium sulfate precipitation, Lane C: Pooled active fractions from Q-Sepharose, Lane D Zymogram analysis by Congo red. Lane B-D shows protein band with molecular weight ~38KDa. Zymogram analysis demonstrates the ability of the extracted enzyme to degrade cellulase

3.4 Effect of pH on Enzyme Activity and Stability

The effect of pH ranging from 3-11 on purified cellulase enzyme from *S.maltophilia* was analyzed by incubating the enzyme with respective buffer and above-mentioned standard assay conditions were followed. The purified enzyme showed maximum activity at pH 8.0 (Fig-5). The enzyme retained its 60-80% activity at pH 7.0 and pH 8.0 for 24 hours, but only 20% at pH 5.0 & 6.0 (Fig-6). This result is in agreement with the previous report of Iqbal *et al.*, (2011) [35] who reported pH-8.0 as an optimum pH for cellulase enzyme production from *Trichoderma viride*.

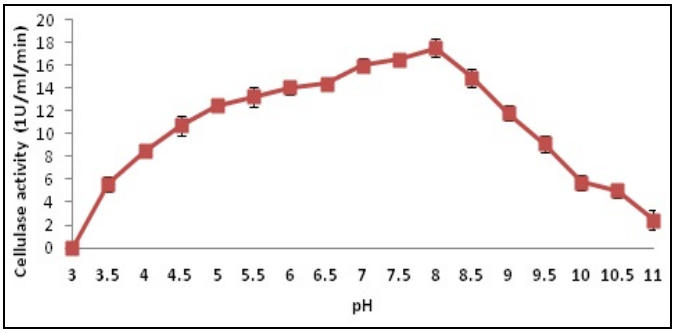


Fig 5: Effect of pH on enzyme activity. Higher activity was observed at the pH 8.0.

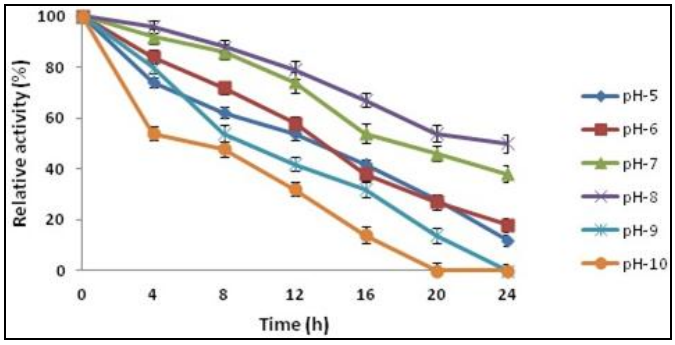


Fig 6: Effect of pH on enzyme stability. The purified enzyme was stored at different pH and observed for its stability in enzyme activity. The enzyme was stable for more than 24 hours at pH 8.0

3.5 Effect of Temperature on Enzyme Activity and Stability

To investigate the effect of temperature on the enzyme activity, the enzyme was incubated at different temperatures (20-100ºC) and estimation of enzyme activity was performed as mentioned previously. The optimal temperature for the purified cellulase was 50ºC and it was stable between 30ºC-50ºC (Fig-7). The enzyme activity declined rapidly above 70ºC. The thermal stability study revealed that at 10ºC the enzyme exhibited 100% of activity even after 24 hours of incubation (Fig-8). This present study correlates well with the earlier reports of *Bacillus* strain PC-BC6 and mutant *Bacillus* N3(Abdullah *et al.*, 2016) [45]. *Streptomyces* C188 (Abdul *et al.*, 2014) [46]. *Paenibacillus terrae* ME27-1(Liang *et al.*, 2014) [47], *Bacillus* strain (Sadhu *et al.*, 2013) [48], *Streptomyces* sp. SLAB-08 (Macedo *et al.*, 2013) [49] that have shown their optimal temperature at 50ºC.

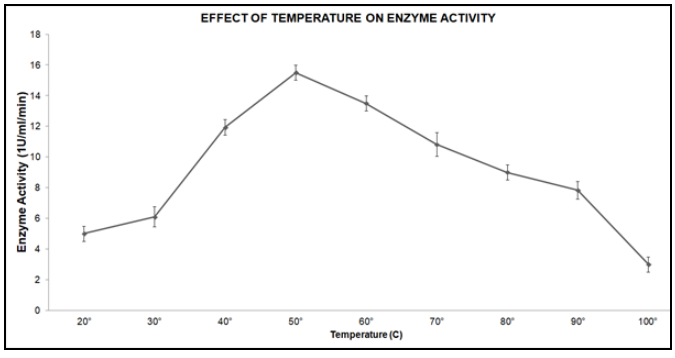


Fig 7: Effect of Temperature on enzyme activity. To find the effect of temperature on enzyme activity, it was measured at temperature range of 20ºC to 100ºC. Higher activity was observed at 50ºC

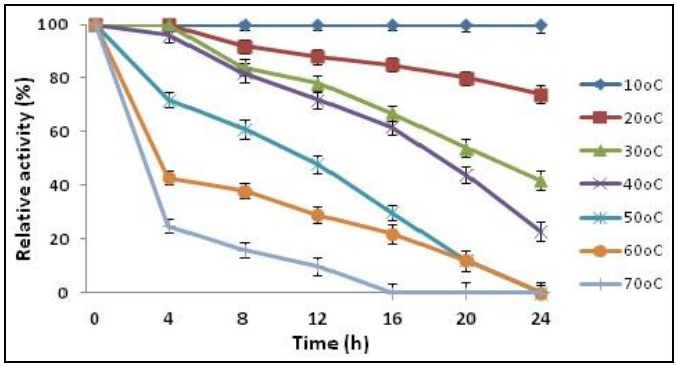


Fig 8: Effect of Temperature on enzyme stability. Enzyme activity was observed to be stable at the temperature of 10ºC for more than 24 hours.

3.6 Effect of Salt Concentration on Enzyme Activity and Stability

Effect of NaCl concentration on the enzyme activity was estimated by pre-incubating the enzyme with different concentration (5-25%) of NaCl at 50ºC for 30mins. Enzyme activity assay revealed that the optimal concentration of NaCl was 5% (Fig-9) and enzyme retained its 90% activity at the same concentration. Enzyme lost its activity as concentration increased from 20-25%. Enzyme stability remained 50-90% stable at 0-15% of NaCl after 24 hours of incubation (Fig-10). It was already reported that cellulase *Salinivibrio* sp.NTU-05 retained its maximum activity after 24 hours incubation in 5% NaCl by Wang *et al.*, (2009) [40]. High salt tolerating cellulase enzyme is of major biotechnological interest for a long period of incubation (Margesin & Schinner 2001) [50].

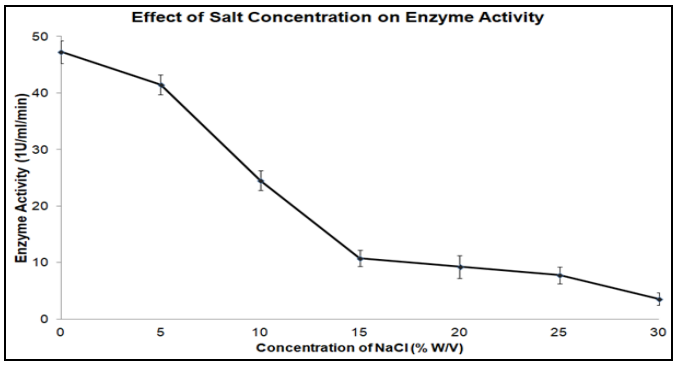


Fig 9: Effect of NaCl concentration on enzyme activity. The enzyme activity is not significantly altered event at 5% of salt concentration and tolerated up to 15% salinity.

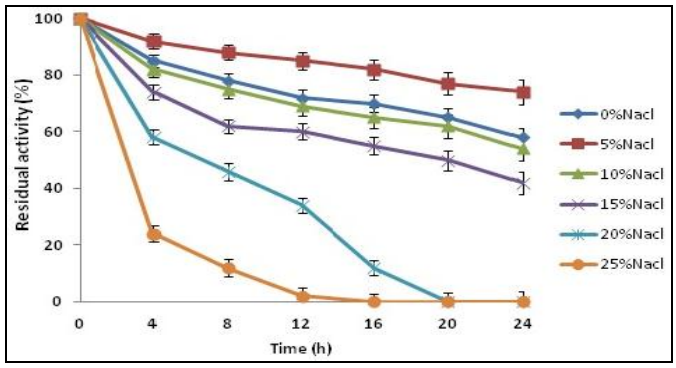


Fig 10: Effect of NaCl concentration on enzyme stability. More than 80% activity was retained for more than 24 hours at the 5% salt concentration.

3.7 Effect of Additives on Enzyme Activity

The effect of 5mM of metal ions on enzyme activity was investigated by allowing the individual metal ions to react with the cellulase enzyme for 30mins at 50ºC followed by normal assay protocol. Enzyme activity was found to be enhanced by Co^{2+}, Na^{+}, K^{+} & Zn^{2+}, while the activity got decreased by Hg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} & Mg^{2+} (Table-2).

Table 2: Different metal ions on enzyme activity

Metal ions (5 mM)	Relative Activity (%)
None	100
Hg^{2+}	42 ± 0.13
Ca^{2+}	80 ± 0.99
Co^{2+}	103 ± 1.15
Cu^{2+}	86 ± 0.70
Fe^{2+}	94 ± 1.14
Mg^{2+}	91 ± 0.54
Mn^{2+}	70 ± 1.16
Na^{+}	97 ± 2.27
K^{+}	110 ± 1.5
Zn^{2+}	100.5 ± 0.47

Previously Ng *et al.*, (2009) [51] and Tao *et al.*, (2010) [52] reported that endoglucanase from *Geobacillus* sp. 70PC531 and *Aspergillus glaucus* XC9 was activated by Co^{2+} and Zn^{2+}. Lima *et al.*, (2005) [53] also described that the stimulation of endoglucanase from *Bacillus pumilis* by Sodium ions. Potassium was also found to enhance the cellulase activity while Hg^{2+} influenced the activity another way around Wang *et al.*, 2009 & Iqbal *et al.*, 2011 [40, 35]. A recent study of Ekwealor *et al.* (2017) [54], reported that apart from Hg^{2+}, Cu^{2+} & Fe^{2+} also inhibit the activity of CMCase from *Bacillus sphaericus* CE-3. To add to the list, Ca^{2+} & Mn^{2+} also inhibited the activity of an alkali-halotolerant cellulase from *B. flexus*.

An effect of organic solvents on the activity of purified cellulase was examined by incubating the enzyme with different organic solvents (5%v/v). The relative enzyme activity remained 100% in the presence of isopropanol, methanol, and ethanol and partially inhibited by toluene (80%) and acetonitrile (63%). Whereas, it was found to decrease in the presence of benzene (61%), Acetone (52%) and Butanol (51%) respectively (Fig-11). Ethanol and isopropanol were previously reported to un-alter the cellulase enzyme activity (Voget *et al.*, 2006 Trivedi *et al.*, 2011) [55, 56].

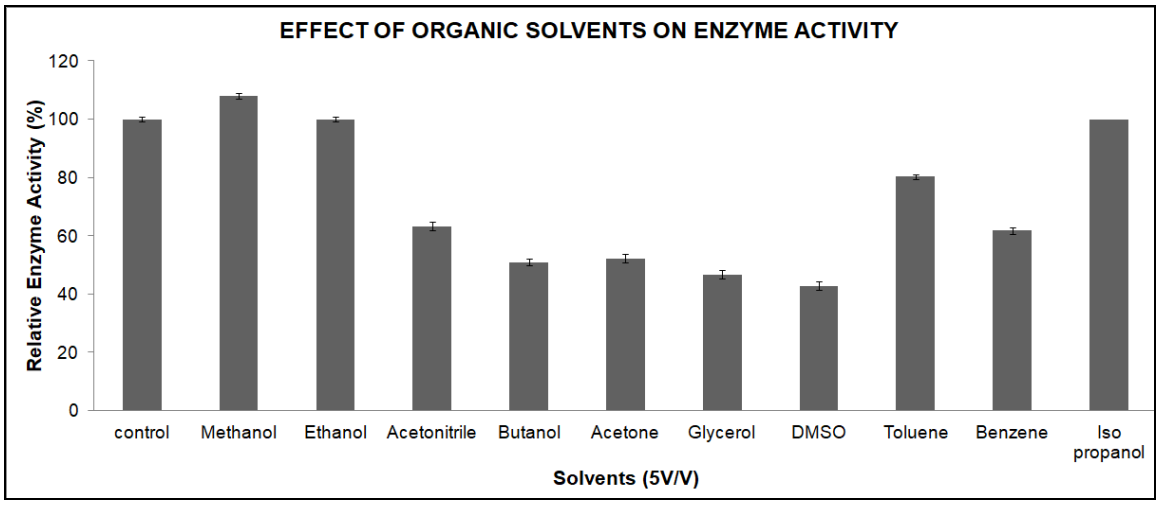


Fig 11: Effect of Organic solvents on enzyme activity. Methanol was found to slightly enhance the enzyme activity while other solvents like butanol and DMSO decreased it.

3.8 Effect of Inhibitor, Chelator, and Denaturant

The effect of β-mercaptoethanol, EDTA, and Urea on the activity of cellulase at 1mM and 10mM concentrations was analyzed. The enzyme was pre-incubated with above-mentioned reagents for 30mins at 50ºC and activity was measured under standard assay conditions. Urea, β –mercaptoethanol and EDTA at 10mM concentration was able to inhibit 35.5%, 22.74% and 20.52% of the cellulase activity (Table-3) while at 1mM concentration it has no significant inhibitory effect. Moderate inhibition by EDTA on enzyme activity has already reported in cellulase from *Bacillus amyoliquefaciens* DL-3 (Lee *et al.*, 2008) [57] and *B. flexus* (Trivedi *et al.*, 2011) [56].

Table 3: Effect of different chemicals on enzyme activity.

Chemical	Cellulase activity (%)
Control	100
Urea (1mM)	84.66 ± 1.64
Urea (10mM)	64.50 ± 1.28
EDTA (1 mM)	84.58 ± 0.58
EDTA (10 mM)	79.48 ± 1.42
β-mercaptoethanol (1 mM)	80.54± 1.25
β-mercaptoethanol (10mM)	77.26 ± 0.75

3.9 Effect of Detergents

The effects of ionic and nonionic detergents on cellulase activity were studied in optimal standard assay conditions. In the presence of surfactants, the enzyme activity was increased

with SDS (113.35%), Triton X100 (112.24%) and Tween 20 (101.28%) at the 0.1% level (Table-4).

Table 4: Effect of detergents on enzyme activity

Detergents	Relative activity of Soluble Cellulase (%)
Control	100.00
Tween 20 (0.1%)	101.28± 0.94
Tween 20 (1%)	98.34± 1.31
Triton X 100 (0.1%)	112.24 ± 0.67
Triton X 100 (1%)	104.90 ± 1.24
SDS (0.1%)	113.35 ± 1.21
SDS (1%)	109.94 ± 0.47

A recent study by Potprommanee *et al.*, (2017)[44] showed that, the thermophilic cellulase from *Geobacillus sp*. HTA426 activity was influenced by ionic and nonionic surfactants like SDS, Triton X 100 and Tween 80. The increase in cellulase activity with these same detergents (SDS, Triton x 100 & Tween 20) has also been observed by Asha *et al.*, (2012) [58]. Compared to 0.1% of surfactants, 1% showed lesser enhancement of enzyme activity. The surfactants were able to modify the surface property and help to minimize the irreversible reaction of cellulase as reported (Wu and Ju 1998) [59].

3.10 Determinations of Kinetic Parameters

The Michaelis-Menten kinetic constants Km and Vmax of purified cellulase were determined using different concentrations of Carboxymethyl cellulose. Enzyme activities were measured under standard assay conditions as described above and cellulase activity (U/ml/min) against the concentration of CMC (mg/ml) was plotted (Fig-12).

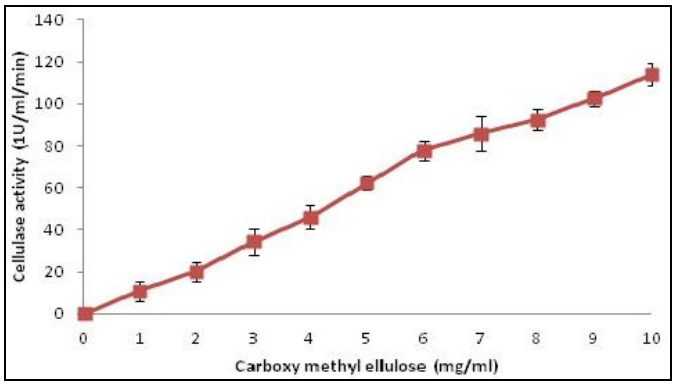


Fig 12: Effect of substrate concentration on enzyme activity. The concentration of the substrate has directly proportional activity on the activity of the enzyme.

The Lineweaver-Burk double-reciprocal (Fig-13) plot was used to determine the Km & Vmax values. The Km of cellulase was 5.41 mg/ml, and Vmax was 161.29 µmol/min/mg. The kinetic parameters obtained from *Stenotrophomonas* sp., are lower than other researchers observed for *B. flexus* (Trivedi *et al.*, 2011) [56] and *Malanocarpus* (Kaur *et al.*, 2007) [60]. Therefore cellulase from *Stenotrophomonas maltophilia* JS-R001 has more affinity towards the substrate**.**

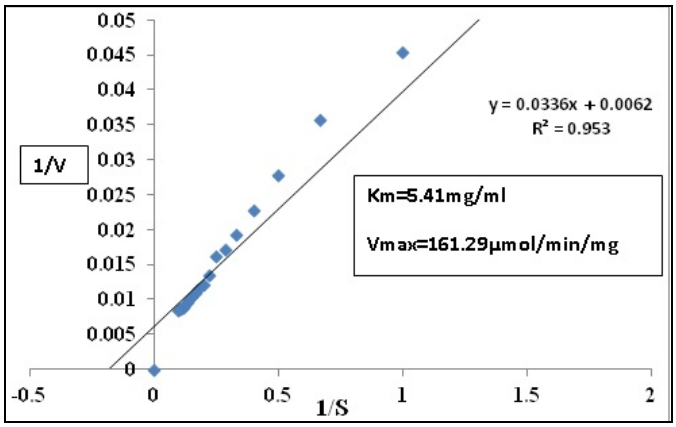
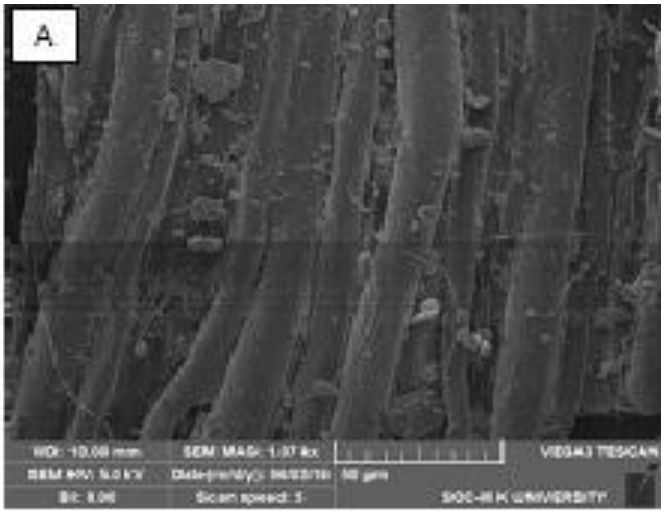


Fig 13: Double reciprocal plot for determining the Vmax & Km values of the cellulase enzyme. The Km value and Vmax value were found to be 5.41mg/ml and 161.29 µmol/min/mg.

3.11 Cellulase for the Degradation of Lignocellulosic Biomass

In the present study, cellulosic substrates like rice straw and sorghum stubbles were treated with purified cellulase from *S.maltophilia* at the concentration of 29.37U/g at 50ºC for 2 hours (pH-8). At 50X magnification under the SEM, significant changes were observed in the substrates treated with the purified cellulase (Fig-14). A short mesh of substrates appeared on the surface of the treated material as an evidence of hydrolysis into simple sugars and hemicelluloses and signifying a tremendous decrease in the complexity of the residual lignin. This property of our purified cellulase from *S. maltophilia* favors its unique applications in biofuel production, degradation of agricultural wastes for biobleaching, in paper and pulp industries.



SN

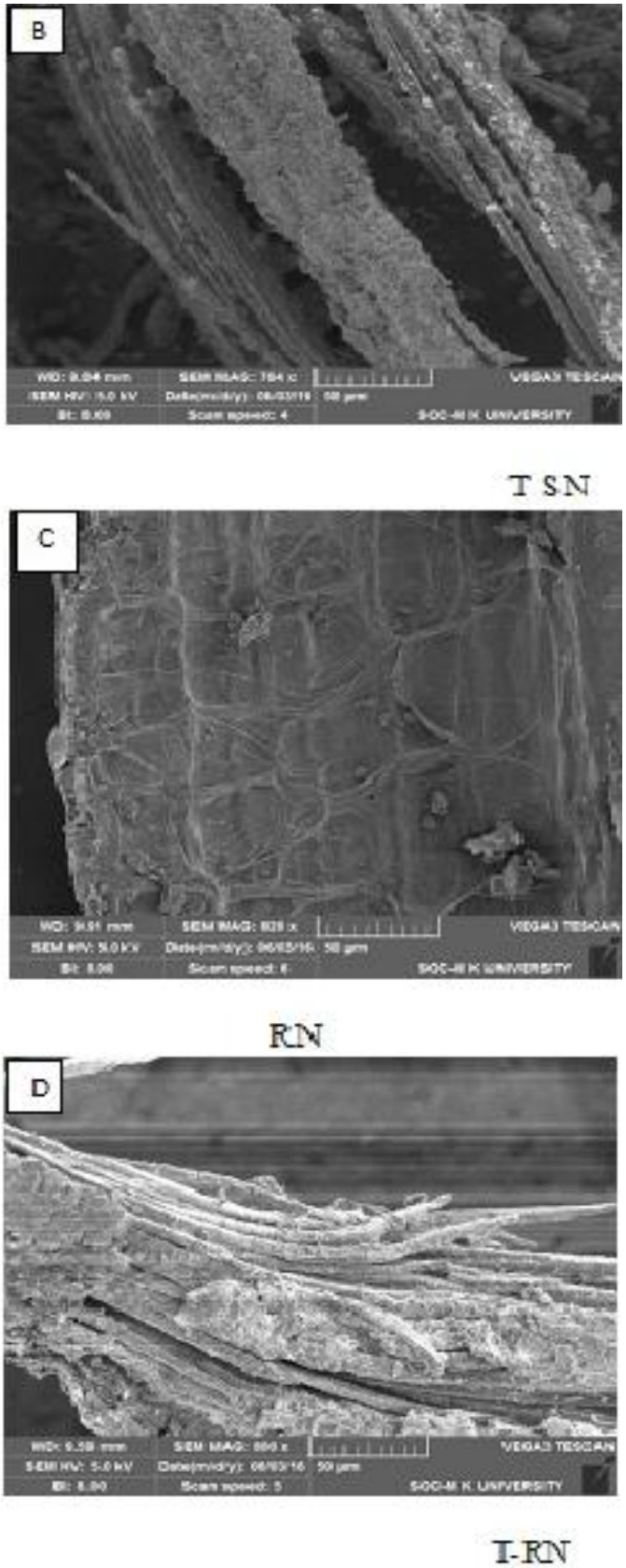


Fig 14: Cellulose degrading potential of Purified Cellulase enzyme shown in SEM images (50X) of substrates (A: Sorghum stubbles, B: Treated Sorghum stubbles, C: Rice straw & D: Treated Rice straw) treated with partially purified enzyme at concentration 29.37 U/g at 50ºC for 2 hrs (pH- 8). Control substrates were washed in buffer before SEM analysis.

On the whole, *Stenotrophomonas maltophilia* was isolated from biogas slurry samples, and it showed good cellulolytic activity. The cellulase enzyme obtained from *S. maltophilia* was found to be alkali-halo tolerating in nature. The optimum temperature and pH of the enzyme activity was identified as 50ºC and pH 8.0 respectively. The enzyme activity was boosted by several additives and detergents like Co^{2+}, Na^{+}, K^{+}, Zn^{2+}, SDS, Triton X-100 and Tween-20. In addition, cellulase from *S.maltophilia* has the ability to degrade complex agro waste materials into simpler forms. This feature indicates potential applications in the biofuel, food, textile industries, and waste treatment.

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