Original Research Article

Measurement of Cellulolytic Potential of Cellulase Producing Bacteria

Abstract:

The bioconversion of cellulose and hemicellulose to soluble sugars is important for global stabilization and for a sustainable human society. Here, hundreds of cellulolytic bacteria were found in soil, compost and animal waste slurry of our environment.

Bacillus sp. are aerobic cellulolytic bacteria. Here, two bacillusstrains 2414, 2579 (T) and its mixed culture utilized for measuring the cellulolytic potential.

The capability of cellulolytic potential was analyzed by enriching the basal salt media with Whatman no.1 filter paper as substrate for cellulose degradation. Here, Cellulose-degrading potentialof *Bacillus* strains was measured by measuring the diameter of clear zone around the colony and its hydrolytic value on cellulose Congo-Red agar media.

The extracellular cellulase activities ranged from 0.08233 to 0.44 IU/mL for FPase and 0.243 to 0.595 IU/mL for endoglucanase assay. The maximum activitiesrange of β-glucosidase (or cellobiase) activity was0.6 to1.5 1U/ml. The maximum xylanase activities values *Bacillus cellulolysticus* 2579 (T), *Bacillus subtitlis*2414 and its mixed culture(12.0,11.5 and 12.5 unit/mL, resp.). All the enzymes were stable at an optimum pH range valueof (3.0-7.0) and temperature range of (30˚C-50˚C).The maximum filter paper degradation percentage was estimated to be 71.76% by mixed culture after 48hrs of incubation period, it was observed that the maximum filter paper degradation was done by mixed culture than *Bacillus* strains.

Biodiesel production was estimated by following EN-14103 method and ester content was calculated on the basis of response factor with a minimum set value of ester content would be 96.5with%.

Key words:cellulase, cellulolytic bacteria, FPase,endoglucanase assay,β-glucosidase, xylanase

List of Abbreviations:

Weight- (gm)

Volume – (L)

Weight/volume-(gm/l)

Mililiter-(ml)

Temperature-(˚C)

Molarity-(M)

Time–(mins, hrs)

Microgram- (µg)

Nanometer-(nm)

Percentage- (%)

Micromole-(µm)

Absorbance- (Abs)

p-Nitrophenol-β-D-glucopyranoside-(*p*-NPG)

Endo-β-1,4-glucanase-(EGase Activity)

1,3-Dinitro salicylic acid-(DNS)

Filter paper assay– (Fpase)

Milimetre-(mm)

Weight percentage-(wt%)

International unit per mililitre-(IU/ml)

Micrometer-(µm)

Centimeter-(cm)

Revolution per minute-rpm

1. Introduction:

Dependence on petroleum based fossil fuel is not sustainable due to its cost, serious environment concern problems and the steady depletion of crude oil[1][2][3].

The solution of this arising problem is to produce renewable fuel: Biodiesel from lignocellulose biomass of agricultural residues, to cut out the usage of fossil fuels in the future.

Lignocellulose biomass of plants are a potential source for the production of biofuels due to its abundance, inexpensive and environmentally friendly nature.

A various agricultural residuewas available for fuel production such as corn fibre, corn stover, sugarcane bagasse, rice hulls, woody crops, coconut residues, and other forest residues.

In addition, dedicated energy crops for biofuels includes: perennial grasses such as Switchgrass and other forage feed stocks such as *Miscanthus*, Bermuda grass, Elephant grass etc.[4].

Approximately, 70% of plant biomass is locked up in 5 and 6 carbon sugars.

These sugars are found in lignocellulose biomass, which is comprised mainly: cellulose (an unbranched β-1, 4-linked homopolymer of glucose), Hemicellulose (pentose sugar) and lignin (Aromatic polymer).

Here, we mainly concentrateon the core sugar of lignocellulose biomass i.e. cellulose.

The reason behind our main emphasis is that cellulose is easily breakable by cellulases (alkaline enzyme) and easily accessible into fermentable monosaccharide, Glucose, which is a multi-utility product, in a much cheaper and biologically favorable process.

Cellulolysis is a basic biological process operated and processed by many microbial enzymatic systems having a cellulolytic activity.

Many microorganisms have been reported with cellulolytic activities including many bacterial and fungal strains both aerobic and anaerobic.

It had been reported that the alkaliphlic*Bacillus* species such as *Bacillus cellulosilyticus*, *Bacillus substils* and *Bacillus sphaericus* producesa higher percentage of extracellular enzymes i.e. cellulase to degrade the cellulose [5][6].

Other cellulolytic bacterial species such as*Clostridium*, *Cellulomonas*, *Thermomonospora*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetivibrio* andActinomycetes in particular *Streptomyces* species[7][8]and *Chaetomium, Fusarium Myrothecium,Trichoderma, Penicillium, Aspergillus,* and so onward,are some of the reported fungal species responsible for cellulosic biomass hydrolysation.

The commercial purpose of producing cellulase is in paper and pulp, textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives [9]. In addition, they find use in saccharification of lignocellulosic agro-residues to fermentable sugars, which can be used for production of bioethanol, lactic acid, and single-cell protein [10].

The aim of the study is to produce alkaline cellulase from *Bacillus* strain and measure the cellulolytic potential of a *Bacillus cellulosilyticus* strain-2579 (T), *Bacillus substilis*-2414 and its mixed culture under optimized physical conditions.

2. **Material and Method**: Bacterial strains: *Bacillus cellulosilyticus*-2579(T) and *Bacillus substilis*-2414 were purchased from NCCS Pune.These bacterial strains were maintained in a nutrient agar slants at 37˚C. All the agar slants were refrigerated at 4˚C and stored for 15 days.

2.1 Screening of cellulolytic bacteria: Primary screening of bacterial strains was performed with a basal salt medium containing filter paper.

Composition of basal salt media($NaNO_3$ 2.5 gm, $KH_2PO_4$2 gm, $MgSO_4$ 0.2gm, NaCl 0.2gm, $CaCl_2.6H_2O$ 0.1 gm in a liter quantity(gm/l).

Confirmation of bacterial strains on a cellulose congo-red agar media by streaking with following composition: $KH_2PO_4$0.5 gm, $MgSO_4$ 0.25 gm, cellulose 2 gm, agar 15 gm, Congo-Red0.2 gm, and gelatin 2 gm; distilled water 1 L (pH 6.8–7.2).

The utility of Congo-Red dye as a sign of cellulolytic bacteria in a cellulose agar media provides the basis of rapid and sensitive screening test for cellulose degrading bacteria. Colonies showing positive discoloration of cellulose Congo-Red media and positive colonies were used for further studies [11].

The cellulose degrading capability by bacteria was defined on the basis of hydrolytic capacity i.e the ratio of diameter of clear zone formed by bacteria to the colony diameter[12].

2.2 Enzyme activity assay:

2.2.1 Endo-β-1,4-glucanase activity:

Endo-β-1,4-glucanase activity was determined by using 0.5ml of supernatant and 0.5ml of 2% amphorous cellulose in a 0.05 M Sodium Citrate buffer pH (4.8) and then incubate in a hot air oven at 50˚C for 30mins.

After 30mins incubation, add 1ml of DNS (1,3-Dinitro salicylic acid) to the 1ml quantity of the reaction mixture and then boiled in water-bath to stop the reaction and take the reading at 510nm on a Systronics uv-spectrophotometer to estimate the reducing sugar.

One unit of endo-β-1,4-glucanase activity was defined as the amount of an enzyme that could hydrolyze Cellulose and release 1µg of glucose within 1 min reaction at 50°C [13].

2.2.2 Filter paper assayactivity (FPase activity):

Filter paper assay activity was determined by adding 0.5ml quantity of supernatant of bacterial culture~50µl with 0.5 ml quantity of 0.05 M Sodium Citrate buffer pH (4.8) (Sodium Citrate buffer pH (4.8) by mixing 9.9ml quantity of sodium citrate with 11.1 ml quantity of citric acid) containing Whatmann No.1 filter paper strips (0.05 gm for 20ml), incubate at 50 ˚C for 1hr. After a 1hr incubation period, add a 3ml quantity of DNS reagent to 1ml reaction mixture to stop the reaction. 100mg quantity of glucosewas required as a standard to estimate the reducing sugar by following spectrophotometer method [14].

2.2.3 Xylanase activity:

Xylanase activity was measured by using Beechwood xylan as substrate [15].

The reaction mixture containing 0.2ml of crude enzyme, 0.5mL of 1% xylan solution in 0.05M phosphate buffer (pH 6.0), and 0.3mL of buffer (pH 6.0) was incubated at 50◦C for 10 min.

The enzymatic reaction was stopped by adding 3mL of DNS reagent, boiled in capped glass tubes for 5 min, and cooled in cold water for color stabilization. The resulting optical density was measured at 520 nm. D-xylose was used as a standard for the preparation of a calibration curve. One unit of xylanase activity was defined as the amount of enzyme that released 1μmol of reducing sugars as xylose equivalents min^{-1}.

2.2.4 β-glucosidase activity:

β-glucosidase activity was determined by the hydrolysis of p-Nitrophenol-β-D-glucopyranoside (*p*-NPG) in 100mM acetate buffer (pH 5.0) at 37°C for 20 min. The reaction was terminated by the addition of 1ml of 0.25M Na_2CO_3. The absorbance (Abs) was measured at 405 nm.

One unit of β-glucosidase was defined as the amount of enzyme that could hydrolyze p-NPG and liberate 1µmol of p-nitrophenol within 1min reaction at 37˚C [16].

2.3 Fermentation of carbohydrate:

Fermentative degradation of various carbohydrates such as glucose, sucrose, cellulose by bacteria under aerobic condition was carried out in a culture tube that contains a Durham tube (i.e. a small tube placed in an inverted position in the culture tube) for the detection of gas bubbles, as am end product of metabolism[17].

Preparation of fermentation media: Peptone 10.0gm, Carbohydrate (D-glucose or Dextrose) 5.0gm, NaCl 15.0gm, Phenol red (pH indicator) 0.018gm and maintain the pH=7.3 for a 1litre.

Later in the preparation of fermentation media, broth taken in to culture tubes, autoclave at 12lbs pressure for 15 mins. Inoculate the bacterial culture in prepared sugar fermentation media (three per culture) and keep one culture tube uninoculated as a control. Incubate all the culture tube at 37˚C for 24-48hrs. From this test, it clearly indicates that the production of organic acids by changing the color from red to yellow.

2.4 Ester content estimation in a Biodiesel:

EN 14103 method was generally useful for an estimation of ester content in biodiesel free of heptadecanoate ester (C17:0) or methyl nonadecanoate as an internal standard.

This method is useful for a whole range of fatty acid (C6-C24:1) methyl ester have been taken into consideration for the calculation of the ester content.In the biodiesel specification, minimum set value of ester content would be 96.5wt%.

The response factor for both the saturated and unsaturated were in the range of 0.97-1.16.

The ester content was calculated after applying the response factor of each methyl ester [18]. The result obtained by this method shows good correlation R^2=0.98 with 1H-NMR method.

3. Result and Discussion:

3.1 Screening of cellulolytic bacteria:

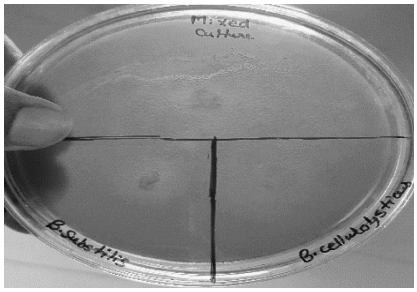
Table.1Morphological and Physiological Characteristics of Cellulolytic Bacteria:*Bacillus subtilis* and *Bacillus cellulosilyticus*.

Characteristics	***Bacillus subtilis*-2414**	***Bacillus cellulosilyticus*-2579 (T)**
Gram staining	+	+
Motility	+	+
Catalase	+	+
Cell shape	Rod	Rod
Size (L, µm)	2.5-3.0 µm	2-3 µm
Colony Appearance		
Shape	Round	Circular
Margin	Entire	Entire
Elevation	umbonate	umbonate
Growth Temperature Range ˚C	28-40 ˚C, optimum (37 ˚C)	30-40 ˚C, optimum (37 ˚C)
Growth pH Range	6.8-7.2	8-10, optimum 9.7

All the bacterial culture was show growth in a basal salt media containing filter paper after 24-96hrs constant shaking at 100rpmon a rotary shaker at 37˚C. After continuous shaking, medium turns cloudy and the filter paper becomes more macerated as the growth of pure culture of *Bacillus substilis*,*Bacillus cellulosilyicus* and mixed culture progress.

Bacterial culture has the potential to degrade the cellulose and release the monosaccharide i.e. glucose. A cellulose degradation was checked by measuring the clear zone around the colony and hydrolytic capacity in a cellulose Congo-Red agar media.

198 All the bacterial culture was show growth in cellulose Congo-Red agar media producing clear
199 zone under aerobic condition as shown in fig.1



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201 Figure.1 Zone of clearance on cellulose Congo Red agar plates for bacterial strains after 24hrs of
202 incubation. The formation of clear zone around colony confirms the extracellular secretion of
203 cellulase.

204 Table:2 Maximum Clearing zone and Hydrolytic capacity (HC) of Bacillus species and its mixed
205 culture on cellulose Congo Red agar media.

Bacterial species	**Maximum Clearing Zone (mm)**	**Average HC value(mm)**	**Maximum HC Value(mm)**
Pure cultureof *Bacillus substilis*-2414 (BS)	15 mm	3.67	1.5
Pure cultureof *Bacillus cellulosilyticus*-2579 (T) (BC)	47mm	1.74	2.24
Mixed culture of (BS)+(BC) strains	48 mm	2.16	2.9

206 Table.2 depicted the potential of bacterial strains was effective against cellulose by measuring
207 the clearing zone and hydrolytic capacity in cellulose Congo Red agar media.
208 The results showthe maximum clearing zone and maximum hydrolytic capacity (HC) value
209 ranged in between 15mm-48mm for pure culture of *Bacillus* strains and their mixed culture.
210 Maximum clearing zone of 48mm and hydrolytic capacity (HC) value of 2.9mm were estimated

for Mixed culture of (BS)+(BC) strains. The range of HC value obtained is similar to range reported by[19]whereas [20] found the hydrolytic value between 1.38 to 2.33 and 0.15 to 1.37 of cellulolytic aerobic bacterial isolates from farming and forest soil, respectively.

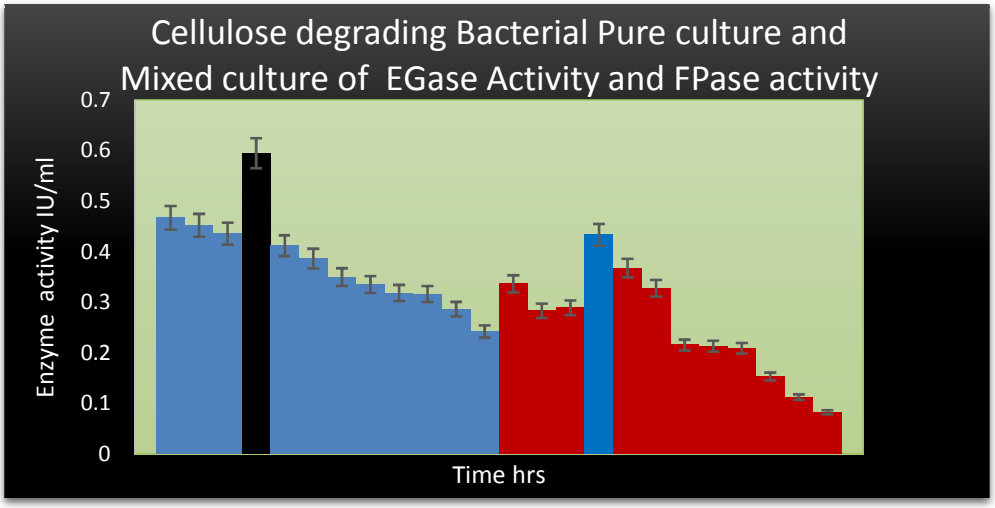

Figure 2: Endoglucanase activity (EGase Activity) and Filter paper activity (FPase activity) were shown at different time interval hours (24,48,72 and 96hrs) of pure culture*Bacillus*subtilis (BS), *Bacilluscellulolysticus* (BC)and its mixed culture. The maximum value for endoglucanase activity (EGase Activity) was 0.595IU/ml (Black bar) whereas the maximum value for filter paper (FPase activity) was 0.44 IU/ml. Values in figure are means of three replicates with standard deviation.

3.2 Assessment of cellulose decomposition potential:

In the present study, shows the cellulolytic potential comparison between a pure culture of *Bacillus* species and their mixed culture. A cellulolytic potential was estimated on the basis of an enzyme production and maximum cellulolytic activity. An enzyme assay for cellulase activity on filter paper was found to be highest for 0.44 IU/ml while endoglucanase assay maximum activity was determined to be 0.595IU/ml.An optimum pH value of 7.0 the FPase enzyme activities ranged from 0.083 to 0.44 IU/mL and 0.243 to 0.595 IU/mLfor endoglucanase assay.Previously reported data showed that alkalinepH range was suitable for endoglucanase activity on production from *Bacillus pumilus*atpH 8.5 endoglucanase activity slightly higher as compared to pH 6.5 and all the experiment was conducted at 37˚C [21].But in this study showed that the

FPase and endoglucanase enzyme activity were stable at temperature range of 30-50˚C and at the pH range of 3.0 to 7.0.

Fig.2a  Fig.2b 

Figure.2a represents the filter paper degradation by *Bacillus cellulolysticus*-2579(T)(BC) (at Left), *Bacillus subtilis*-2414 (at Right) and mixed culture of *Bacillus* strains (at middle)cultured in basal salt medium with Whatman no.1 filter paper strips (1.0×6.0cm) at the end of 96hrs of incubation with constant shaking at 100 rpm on rotating shaker.

Fig.2b is the control where no filter paper degradation takes place.

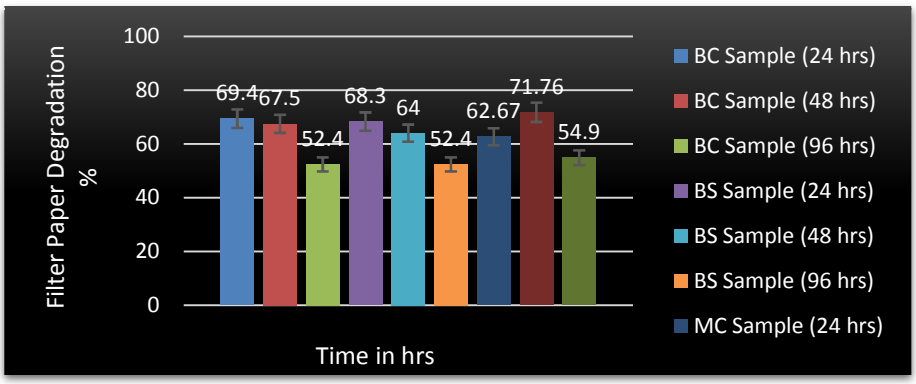

Figure.3 shows the percent filter paper degradation by pure cultures of *Bacillus cellulolysticus* (BC Sample), *Bacillus subtilis* (BS Sample) and Mixed Culture of BC and BS strains by Spectrophotometric method.Maximum percentage of filter paper degradation was found to be 71.7% by mixed culture after 96hrs incubation period. Values shown in the figures.3 are means of three replicates with Standard deviation.

[22] also reported that endo-*β*-1,4-glucanases from alkalophilic *B. subtilis* strains 1139 and N-4 were capable of hydrolyzing CMC, but could not degrade Avicel significantly. Our study also reports the same results. [23]have described an enhancement of cellulose degradation with coculture consisting of the aerobic cellulolytic fungus *Tricoderma harzianum* and the anaerobic bacterium *Clostridium butyricum*.Previous studies by [24] also reported that *Enterobacter* and *Acinetobacter* species showed cellulolytic activity.

In present study, shows the maximum activities of β-glucosidase (or cellobiase) activity were 1.0, 0.6, 1.5 for 2579 (T),2414 and mixed culture of bacillus strains in unit/mL respectively after incubating for 24hr at 37˚C and further analyzed at same temperature but for different incubation period.

The enzyme activities, subsequently decreased after reaching maximum values and then second rises and falls were observed. However, all the *Bacillus* strains showed considerable β-glucosidase activity in the cell debris fraction.

A reason for the rise and fall in β-glucosidase activity might be the negative regulation of β-glucosidase gene expression by glucose level (catabolite repression) in the cells.

β-glucosidase has scarcely been reported in *Bacillus* strains, [25] found that *B. licheniformis* could grow in minimal media containing cellobiose, but failed to show the presence of cellobiasein either the cellular fraction or culture supernatant

Although, β-glucosidase production by other bacteria like *Clostridium thermocellum* [26]and *Alcaligenesfaecalis* [27]had been documented.

The *Bacillus* strains 2579 (T) and 2414 showed considerable xylanase activity in the cell-free culture supernatant, and their activities reached to maximum values (12.0, 11.5 and unit/ml resp.) after shaking culture at 100 rpm for 96hrs with 10 gm/litreof Carboxy-methylcellulose as a carbon source and no xylanase activity was detected in the cell debris fraction.

Xylanase production has been previously reported in *Bacillus* strains [28].

[29] reported that all examined cellulolytic *Bacillus* species were also xylanase positive, and units of xylanase activity were found to be much higher as compared to corresponding CMCase activity units.

All the *Bacillus* sp. showed a potential to convert cellulose into reducing sugars, which could be readily used in many applications like feed stock for production of valuable organic compounds as well as strains are presently being employed in agriculture as a fertilizer supplement.

They especially were quite effective as ingredients of an organic seed bed.

4. Conclusion:

In country like India, where population already reached billion, cutting or managing usage of crude oil based fuels or finding the biofuels based alternative is an urgent need over fossil fuels. Cereal grains or edible oil

based biofuels are also not affordable, as they are food commodities. Under these circumstances, abundant lignocellulose based plant biomass is the best available option to be explored for production of biofuels like bioethanol or biodiesel etc.

Here, we utilized the pure culture of Bacillus strains:*Bacillussubtitlis*-2414, *Bacillus cellulolysticus*-2579 (T) and its mixed culture to measure the cellulolytic potential on the basis of biochemical assay.The cellulolytic potential was measured by filter paper degradation, maximum degradation of up to 71.76% by mixed culture after 48hrs of incubation period.

This property of cellulolytic potential was determined by calculating the hydrolytic value (HC Value) on cellulose congo-red agar media.

Both the bacterial strains have an ability to dissolve the complex sugar i.e. cellulose and break into the reducing sugar i.e. glucose and the released reducing sugar which was later used for biofuel production, which in turn would also increase biofuel production.

5. References:

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