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 asubstrate for cellulose degradation. Here, Cellulose-degrading potentialof *Bacillus* strains was measured by measuring the diameter of aclear 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 activitiesvalue*Bacilluscellulolysticus*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 theEN-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%.

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

List of Abbreviations:

Weight- (gm)

Volume – (L)

Weight/volume-(gm/l)

Milliliter-(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.

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Lignocellulose biomass of plants is 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 fiber, 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.[4-5].

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-linkedhomopolymer 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 [6][7].

Other cellulolytic bacterial species such as*Clostridium*, *Cellulomonas*, *Thermomonospora*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetivibrio*and*Actinomycetes* in particular *Streptomyces* species[8][9]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 [10].

In addition to commercially usage of cellulase, utilized for the saccharification of lignocellulosic agro-residues into fermentable sugars converted into bioethanol, lactic acid, and single-cell protein [11].

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 and stored at 4˚C.

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

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Composition of basal salt media($NaNO_3$ 2.5gm, $KH_2PO_4$2gm, $MgSO_4$ 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 thefollowing composition: $KH_2PO_4$0.5 gm, $MgSO_4$ 0.25 gm, cellulose 2gm, agar 15gm, Congo-Red0.2 gm, and gelatin 2gm; 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 arapid 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 [12].

The cellulose degrading capability by bacteria was defined on the basis of hydrolytic capacity i.e the ratio of thediameter of aclear zone formed by bacteria to the colony diameter[13].

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 incubated 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 SystronicsUV-spectrophotometer to estimate the reducing sugar.

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Comment [S14]: After 30 min of incubation, 1 ml of DNS (1,3, Dinitro salicylic acid) was added into 1 ml of reaction mixture and kept in water bath to stop the reaction. Absorbance was measured at 510 nm using 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 [14].

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 the1ml reaction mixture to stop the reaction. The 100mg quantity of glucosewas required as a standard to estimate the reducing sugar by following spectrophotometer method [15].

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2.2.3 Xylanase activity:

Xylanase activity was measured by using Beechwoodxylan as substrate [16].

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 [17].

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 an end product of metabolism[18].

The compositionof 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.

The composition of fermentation media, is followed to make broth culture, autoclaved at 12lbs pressure for 15 mins. Incubate all the culture tube at 37˚C for 24-48hrs. From this test, it clearly indicates that the organic acids were produced if broth culture color were changed from red to yellow.

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Comment [S17]: From this test, it is expected that the production of organic acids, if the reaction mixture turned to yellow from red colour.

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, theminimumset value of ester content would be 96.5wt%.The response factor for both the saturated and unsaturated was in the range of 0.97-1.16.

The ester content was calculated after applying the response factor of each methyl ester [19]. The result obtained by this method shows good correlation R^2=0.98 with the1H-NMR method.

3. Result and Discussion:

3.1 Screening of cellulolytic bacteria:

Table1. Morphological 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 shown the 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 apure 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

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degradation was checked by measuring the clear zone around the colony and hydrolytic capacity in a cellulose Congo-Red agar media.

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

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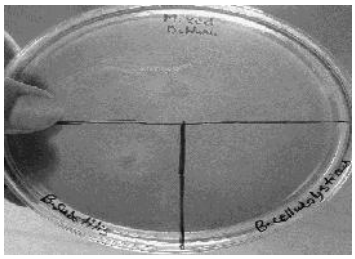

Fig.1.Zone of clearance on Cellulose Congo Red agar plates for bacterial strains after 24hrs of incubation. The formation of aclear zone around the colony confirms thesecretion of cellulaseextracellularly.

Table 2. Maximum Clearing zone and Hydrolytic capacity (HC) of Bacillus strains and its mixed consortium on Cellulose Congo Red agar media.

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Bacterial strains and mixed consortium	**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

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Table.2 depicted the potential of bacterial strains was effective against cellulose by measuring the clearing zone and hydrolytic capacity in Cellulose Congo Red agar media.

The results showthe maximum clearing zone diameter and maximum hydrolytic capacity (HC) ranges from 15mm-48mm for apure culture of *Bacillus* strains and its mixed culture. An observed value of maximum clearing zone diameter was 48mm and the 2.9mm ofhydrolytic capacity (HC) was found for Mixed culture of (BS)+(BC) strain. The obtainedrange of HC value is similar to range reported by[20]whereas [21] found the hydrolytic value between 1.38 to 2.33 and 0.15 to 1.37 by cellulolytic aerobic bacterial isolates from farming and forest soil, respectively.

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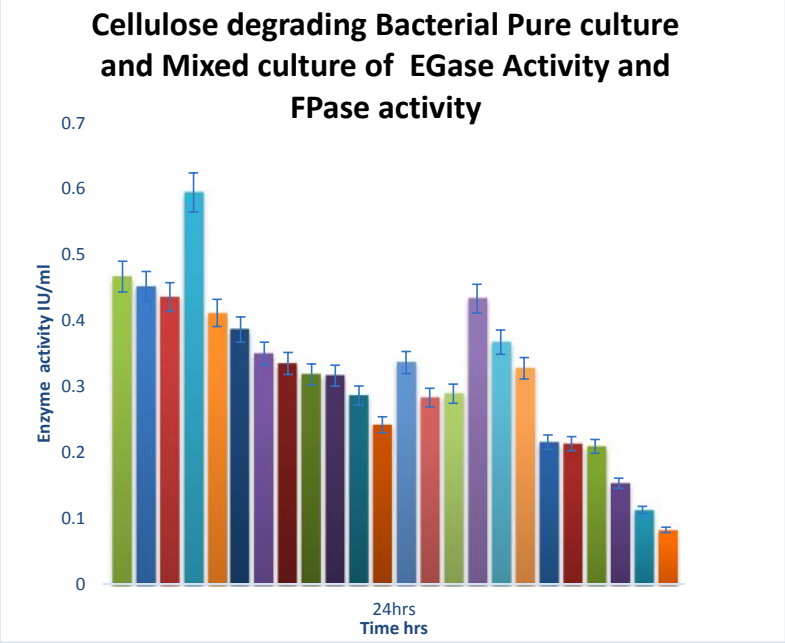

Fig.2. Endoglucanase activity (EGase Activity) and Filter paper activity (FPase activity) were shown at adifferent time interval (24,48,72 and 96hrs) of pure culture: *Bacillussubtilis* (BS), *Bacilluscellulolysticus* (BC)and its mixed culture. The maximum value for endoglucanase

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 thefigure 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 [22].But in the present study showed that the FPase and endoglucanase enzyme activity were stable at atemperature range of 30-50˚C and inthe pH range of 3.0 to 7.0.

Fig.2a  Fig.2b 

Fig.2a.represents the filter paper degradation by *Bacillus cellulolysticus*-2579(T)(BC) (Left flask), *Bacillus subtilis*-2414 (Right flask) and mixed culture of *Bacillus* strains (middle

flask)cultured in basal salt medium with Whatman no.1 (filter paperstrip size of 1.0×6.0cm) kept for 96hrs incubation period with constant shaking at 100 rpm on rotating shaker.

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

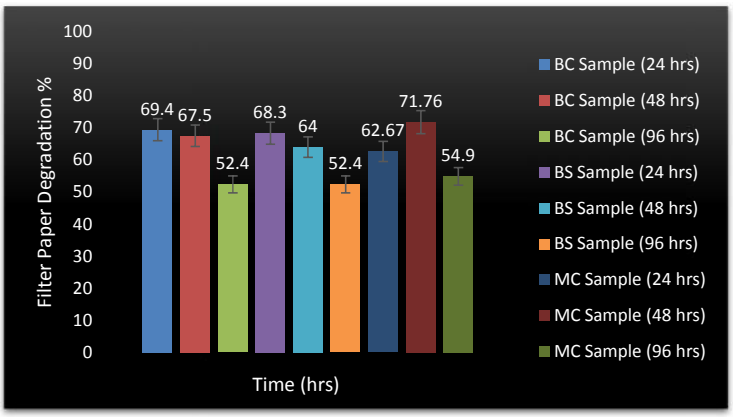

Fig.3.shows the percent filter paper degradation by pure cultures of *Bacillus cellulolysticus* (BC), *Bacillus subtilis* (BS) and mixed culture (BC+BS strains) by theSpectrophotometric method.Maximum observed percentage of filter paper degradation value would be 71.7% by mixed culture at 48hrs incubation period. The values shown in the figures.3 are means of three replicates with standard deviation.

The endo-β-1,4-glucanases excreted from alkalophilic *B. subtilis*strain 1139 and N-4 were capable of hydrolyzing carboxy methyl cellulose (CMC), but could not degrade Avicel significantly[23]. Similar finding was reported in this study.An enhancement of cellulose degradation with coculture with the aerobic cellulolytic fungus *Tricodermaharzianum* and the anaerobic bacterium *Clostridium butyricum*[24] and the *Enterobacter* and *Acinetobacter* species showed cellulolytic activity[25].

In thepresent 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 respectivelyafter

incubating at 37˚C for 24 hrs.Theβ-glucosidase activity of0.038U/mL, by *Bacillus pumilus* EB3 produced in a 2L stirred tank reactor, but the value of β-glucosidase activity is higher as compared to recorded results[26].

The value of enzyme activities, subsequently decreased after reaching maximum values and this rises and falls in enzyme activities were observed.

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.

However, all the *Bacillus* strains showed considerable β-glucosidase activity in the cell debris fraction.β-glucosidase activity has scarcely been reported in *Bacillus* strains, [27] reported that the*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*[28]and *Alcaligenesfaecalis*[29]had been documented also.

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 continuesshaking the culture at 100 rpm for 96hrs with 10 gm/litreof Carboxy-methylcellulose as a carbon source but no xylanase activity was detected in the cell debris fraction. Xylanase production has been previously reported in *Bacillus* strains [30].

[31] 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.

P. curdlanolyticus DSMZ-10248 appears to be xylanolytic bacteria because of its capability to produce xylanase and mannanase in xylan and mannan mediums. The genus *Paenibacillus* was

capable of degrading various polysaccharides and able to secrete multifunctional enzymes, mainly xylanases [32]. The results of this study are also consistent with previous findings concerning *P. curdlanolyticus*, which has been shown to exhibit effective degradation of xylan and cellulose and to produce a multienzyme complex containing several xylanases and cellulases[33].All the *Bacillus* strains were 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 this research study, the cellulolytic enzyme activity of pure culture of *Bacillus sp.*compared with mixed culture of *Bacillus subtitlis*and *Bacilluscellulolysticus*. The pure culture of Bacillus strains:*Bacillussubtitlis*-2414, *Bacillus cellulolysticus*-2579 (T) and its mixed culture were used to measure the cellulolytic potential.

The substrate used by*Bacillus* strains was Whatman filter paper, enriched with basal salt media. The cellulolytic potential was measured by filter paper degradation, maximum degradation of up to 71.76% was done by a mixed culture of bacillus strains after 48hrs of theincubation period. This property of cellulolytic potential was determined by calculating the hydrolytic value (HC Value) on cellulose Congo-red agar media. The basic concept of this study is to analyze the bacterial strains potentially more suitable candidate in terms of degradation of filter paper, indirectly influence the biofuel yield. Based on the result of this study,a mixed consortium of cellulolytic bacterial strains improves the nutrient quality of the substrate and degrade it into reducing sugar: glucose by eliminating crude fibers, which was later used in biofuel production.

5. References:

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