1 **Original Research Article**

2 **Measurement of Cellulolytic Potential of Cellulase Producing Bacteria**

3 **Abstract:**

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

7 *Bacillus* spp. are aerobic cellulolytic bacteria. Here, two *Bacillus* strains 2414, 2579 (T) and their
8 mixed culture utilized for measuring the cellulolytic potential.

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

13 The extracellular cellulase activities ranged from 0.08233 to 0.44 IU/mL for FPase and 0.243 to
14 0.595 IU/mL for endoglucanase assay. The maximum activities range of β-glucosidase or
15 cellobiase activity was 0.6 to1.5 1U/ml. The maximum xylanase activities value *Bacillus*
16 *cellulolysticus* 2579 (T), *Bacillus subtilis* 2414 and their mixed culture were 12.0,11.5 and 12.5
17 unit/mL, respectively. All the enzymes were stable at an optimum pH range value of 3.0-7.0 and
18 temperature range of 30˚C-50˚C. The maximum filter paper degradation percentage was
19 estimated to be 71.76% by mixed culture after 48hrs of incubation period, it was observed that
20 the maximum filter paper degradation was done by mixed culture than *Bacillus* strains.

21 Biodiesel production was estimated by following the EN-14103 method and ester content was
22 calculated on the basis of response factor with a minimum set value of ester content will be
23 96.5%.

24 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%)

44 International unit per mililitre-(IU/ml)

45 Micrometer-(µm)

46 Centimeter-(cm)

47 Revolution per minute-rpm

48 **1. Introduction**:

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

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

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

55 A various agricultural residue was available for fuel production such as corn fiber, corn stover,
56 sugarcane bagasse, rice hulls, woody crops, coconut residues, and other forest residues.

57 In addition, dedicated energy crops for biofuels includes perennial grasses such as Switchgrass
58 and other forage feed stocks such as *Miscanthus*, Bermuda grass, Elephant grass etc. [4-5].

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

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

63 Here, we mainly concentrate on the core sugar of lignocellulose biomass i.e. cellulose.

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

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

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

71 It had been reported that the alkaliphlic *Bacillus* spp. such as *Bacillus cellulosilyticus*, *Bacillus*
72 *subtilis* and *Bacillus sphaericus* produces a higher percentage of extracellular enzymes i.e.
73 cellulase to degrade the cellulose [6] [7].

74 Other cellulolytic bacterial species such as *Clostridium*, *Cellulomonas*, *Thermomonospora*,
75 *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetivibrio* and *Actinomycetes* in particular *Streptomyces*
76 species [8] [9] and *Chaetomium, Fusarium Myrothecium, Trichoderma, Penicillium, Aspergillus,*
77 and so onward, are some of the reported fungal species responsible for cellulosic biomass
78 hydrolysation.

79 The commercial purpose of producing cellulase is for paper and pulp, textile, laundry, pulp and
80 paper, fruit juice extraction, and animal feed additives [10].

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

84 The aim of the study is to produce alkaline cellulase from *Bacillus* strains and measure the
85 cellulolytic potential of a *Bacillus cellulosilyticus*-2579 (T), *Bacillus subtilis*-2414 and their
86 mixed culture under optimized physical conditions.

87 ## 2. Material and Method:

88 Bacterial strains: *Bacillus cellulosilyticus*-2579(T) and *Bacillus subtilis*-2414 were purchased
89 from NCCS Pune. These bacterial strains were maintained in a nutrient agar slants and stored at
90 4˚C.

91 2.1 **Screening of cellulolytic bacteria**: Primary screening of bacterial strains was performed
92 with a basal salt medium containing filter paper.

93 Composition of basal salt media ($NaNO_3$ 2.5gm, KH_2PO_4 2gm, $MgSO_4$ 0.2gm, NaCl 0.2gm,
94 $CaCl_2.6H_2O$ 0.1 gm in a liter quantity (gm/l).

95 Confirmation of bacterial strains on a cellulose congo-red agar media by streaking with the
96 following composition: KH_2PO_4 0.5 gm, $MgSO_4$ 0.25 gm, cellulose 2gm, agar 15gm, Congo-Red
97 0.2 gm, and gelatin 2gm; distilled water 1 L (pH 6.8–7.2).
98 The utility of Congo-Red dye as a sign of cellulolytic bacteria in a cellulose agar media provides
99 the basis of a rapid and sensitive screening test for cellulose degrading bacteria.
100 Colonies showing positive discoloration of cellulose Congo-Red media and positive colonies
101 were used for further studies [12].
102 The cellulose degrading capability by bacteria was defined on the basis of hydrolytic capacity i.e
103 the ratio of the diameter of a clear zone formed by bacteria to the colony diameter [13].

104 **2.2 Enzyme activity assay**:

105 **2.2.1 Endo-β-1,4-glucanase activity**:

106 Endo-β-1,4-glucanase activity was determined by using 0.5ml of supernatant and 0.5ml of 2%
107 amphorous cellulose in a 0.05 M Sodium Citrate buffer pH (4.8) and then incubated in a hot air
108 oven at 50˚C for 30mins. After 30mins incubation, 1ml of DNS was added (1,3-Dinitro salicylic
109 acid) to the 1ml quantity of the reaction mixture and then boiled in water-bath to stop the
110 reaction and take the reading at 510nm on a Systronics UV-spectrophotometer to estimate the
111 reducing sugar.

112 One unit of endo-β-1,4-glucanase activity was defined as the amount of an enzyme that could
113 hydrolyze Cellulose and release 1μg of glucose within 1 min reaction at 50°C [14].

114 **2.2.2 Filter paper assay activity** (FPase activity): Filter paper assay activity was determined by
115 adding 0.5ml quantity of supernatant of bacterial culture~50µl with 0.5 ml quantity of 0.05 M
116 Sodium Citrate buffer pH (4.8) (Sodium Citrate buffer pH (4.8) by mixing 9.9ml quantity of
117 sodium citrate with 11.1 ml quantity of citric acid) containing Whatmann No.1 filter paper strips
118 (0.05 gm for 20ml), incubated at 50 ˚C for 1hr. After a 1hr incubation period, 3ml quantity of
119 DNS reagent was added to the 1ml reaction mixture to stop the reaction.

120 The 100mg quantity of glucose was required as a standard to estimate the reducing sugar by
121 following spectrophotometer method [15].

122 **2.2.3 Xylanase activity**:

123 Xylanase activity was measured by using Beechwood xylan as substrate [16].
124 The reaction mixture containing 0.2ml of crude enzyme, 0.5mL of 1% xylan solution in 0.05M
125 phosphate buffer (pH 6.0), and 0.3mL of buffer (pH 6.0) was incubated at 50◦C for 10 min.
126 The enzymatic reaction was stopped by adding 3mL of DNS reagent, boiled in capped glass
127 tubes for 5 min, and cooled in cold water for color stabilization. The resulting optical density was
128 measured at 520 nm. D-xylose was used as a standard for the preparation of a calibration curve.
129 One unit of xylanase activity was defined as the amount of enzyme that released 1μmol of
130 reducing sugars as xylose equivalents min^{-1}.

131 **2.2.4 β-glucosidase activity**:

132 β-glucosidase activity was determined by the hydrolysis of p-Nitrophenol-β-D-Glucopyranoside
133 (*p*-NPG) in 100mM acetate buffer (pH 5.0) at 37°C for 20 min. The reaction was terminated by
134 the addition of 1ml of 0.25M Na_2CO_3. The absorbance (Abs) was measured at 405 nm.
135 One unit of β-glucosidase was defined as the amount of enzyme that could hydrolyze p-NPG and
136 liberate 1µmol of p-nitrophenol within 1min reaction at 37˚C [17].

137 **2.3 Fermentation of carbohydrate**:

138 Fermentative degradation of various carbohydrates such as glucose, sucrose, cellulose by
139 bacteria under aerobic condition was carried out in a culture tube that contains a Durham tube
140 (i.e. a small tube placed in an inverted position in the culture tube) for the detection of gas
141 bubbles, as an end product of metabolism [18].

142 The composition of fermentation media: Peptone 10.0gm, Carbohydrate (D-glucose or Dextrose)
143 5.0gm, NaCl 15.0gm, Phenol red (pH indicator) 0.018gm and maintain the pH=7.3 for a 1litre.

144 The composition of fermentation media, is followed to make broth culture, autoclaved at 12lbs
145 pressure for 15 mins. Incubated all the culture tube at 37˚C for 24-48hrs. From this test, it is
146 expected that the production of organic acids if the reaction mixture turned to yellow from red
147 colour.

148 **2.4 Ester content estimation in a Biodiesel**:

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

151 This method is useful for a whole range of fatty acid (C6-C24:1) methyl ester have been taken
152 into consideration for the calculation of the ester content. In the biodiesel specification, the
153 minimum set value of ester content would be 96.5wt%. The response factor for both the
154 saturated and unsaturated was in the range of 0.97-1.16.

155 The ester content was calculated after applying the response factor of each methyl ester [19]. The
156 result obtained by this method shows good correlation R^2=0.98 with the 1H-NMR method.

157

158 **3. Result and Discussion**:

159 **3.1 Screening of cellulolytic bacteria**:

160 Table 1. Morphological and Physiological Characteristics of Cellulolytic Bacteria: *Bacillus*
161 *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

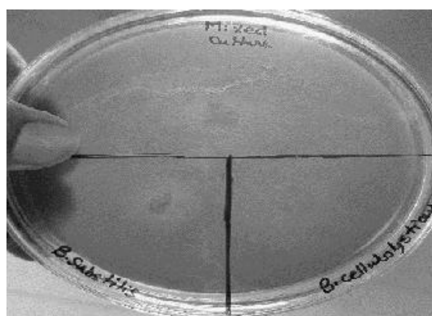
162 All the bacterial cultures were shown the growth in a basal salt media containing filter paper
163 after 24-96hrs constant shaking at 100rpm on a rotary shaker at 37˚C. After continuous shaking,
164 medium turns cloudy and the filter paper becomes more macerated as the growth of a pure
165 culture of *Bacillus subtilis*, *Bacillus cellulosilyicus* strains and their mixed culture progress.
166 Bacterial culture has the potential to degrade the cellulose and release the monosaccharide i.e.

167 glucose. A cellulose degradation was checked by measuring the clear zone around the colony

168 and hydrolytic capacity in a cellulose Congo-Red agar media.

169 All the bacterial cultures were shown the growth in a cellulose Congo-Red agar media producing

170 clear zone under aerobic condition as shown in fig.1



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172 Fig.1. Zone of clearance on Cellulose Congo Red agar plates for bacterial strains after 24hrs of

173 incubation. The formation of a clear zone around the colony confirms the secretion of cellulase

174 extracellularly.

175 Table 2. Maximum Clearing zone and Hydrolytic capacity (HC) of *Bacillus* strains and their

176 mixed consortium of two strains on Cellulose Congo Red agar media.

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

177 Table.2 depicted the potential of bacterial strains was effective against cellulose by measuring

178 the clearing zone and hydrolytic capacity in Cellulose Congo Red agar media.

179 The results show the maximum clearing zone diameter and maximum hydrolytic capacity (HC)

180 ranges from 15mm-48mm for a pure culture of *Bacillus* strains and their mixed culture.

181 An observed value of maximum clearing zone diameter was 48mm and the 2.9mm of hydrolytic

182 capacity (HC) was found for Mixed culture of (BS)+(BC) strain. The obtained range of HC value

183 is similar to range reported by [20] whereas [21] found the hydrolytic value between 1.38 to 2.33

184 and 0.15 to 1.37 by cellulolytic aerobic bacterial isolates from farming and forest soil,

185 respectively.

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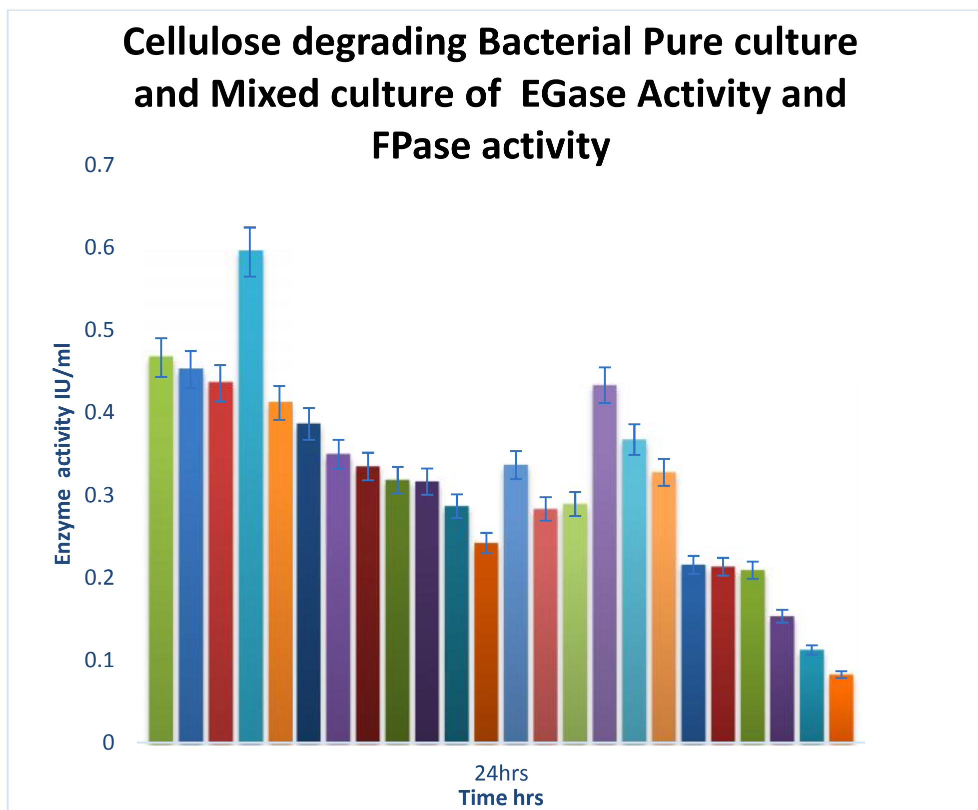


187 Fig.2. Endoglucanase activity (EGase Activity) and Filter paper activity (FPase activity) were

188 shown at a different time interval (24, 48, 72 and 96hrs) of pure culture: *Bacillus subtilis* (BS),

189 *Bacillus cellulolysticus* (BC)and their 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 the 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/mL for endoglucanase assay.

Previously reported data showed that alkaline pH range was suitable for endoglucanase activity on production from *Bacillus pumilus* at pH 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 a temperature range of 30-50˚C and in the pH range of 3.0 to 7.0.

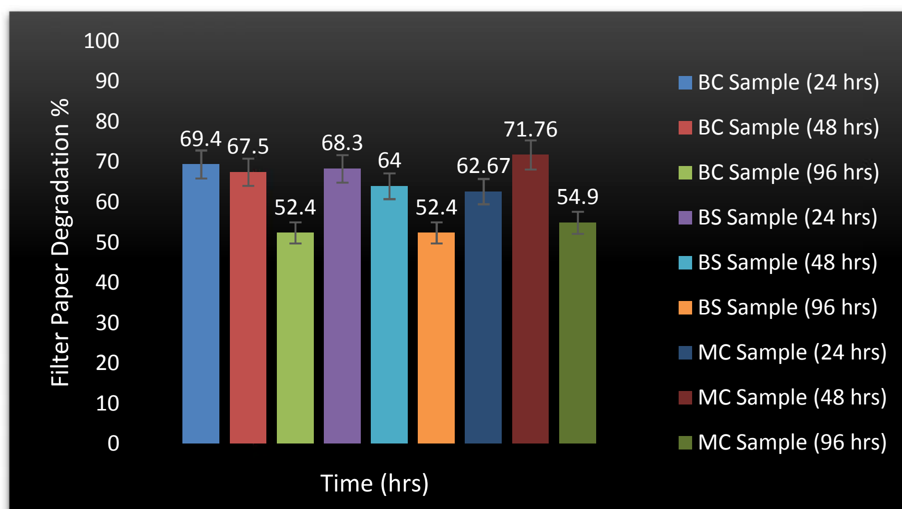
Fig.2a Fig.2b

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)

208 cultured in basal salt medium with Whatman no.1 (filter paper strip size of 1.0×6.0cm) kept for

209 96hrs incubation period with constant shaking at 100 rpm on rotating shaker.

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



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212 Fig.3. shows the percent filter paper degradation by pure cultures of *Bacillus cellulolysticus*

213 (BC), *Bacillus subtilis* (BS) and mixed culture of (BC+BS strains) by the Spectrophotometric

214 method. Maximum observed percentage of filter paper degradation value would be 71.7% by

215 mixed culture at 48hrs incubation period. The values shown in the figures.3 are means of three

216 replicates with standard deviation.

217 The endo-β-1,4-glucanases excreted from alkalophilic *B. subtilis* strain 1139 and N-4 were

218 capable of hydrolyzing carboxy methyl cellulose (CMC), but could not degrade Avicel

219 significantly [23]. Similar finding was reported in this study. An enhancement of cellulose

220 degradation with coculture with the aerobic cellulolytic fungus *Tricoderma harzianum* and the

221 anaerobic bacterium *Clostridium butyricum* [24] and the *Enterobacter* and *Acinetobacter* species

222 showed cellulolytic activity [25].

223 In the present study, shows the maximum activities of β-glucosidase (or cellobiase) activity were

224 1.0, 0.6, 1.5 for 2579 (T), 2414 and mixed culture of *Bacillus* strains in unit/mL respectively

225 after incubating at 37˚C for 24 hrs. The β-glucosidase activity of 0.038U/mL, by *Bacillus*
226 *pumilus* EB3 produced in a 2L stirred tank reactor, but the value of β-glucosidase activity is
227 higher as compared to recorded results [26].
228 The value of enzyme activities, subsequently decreased after reaching maximum values and this
229 rises and falls in enzyme activities were observed.
230 A reason for the rise and fall in β-glucosidase activity might be the negative regulation of β-
231 glucosidase gene expression by glucose level (catabolite repression) in the cells.
232 However, all the *Bacillus* strains showed considerable β-glucosidase activity in the cell debris
233 fraction. β-glucosidase activity has scarcely been reported in *Bacillus* strains, [27] reported that
234 the *B. licheniformis* could grow in minimal media containing cellobiose, but failed to show the
235 presence of cellobiase in either the cellular fraction or culture supernatant.
236 Although, β-glucosidase production by other bacteria like *Clostridium thermocellum* [28] and
237 *Alcaligenes faecalis* [29] had been documented also.
238 The *Bacillus* strains 2579 (T) and 2414 showed considerable xylanase activity in the cell-free
239 culture supernatant, and their activities reached to maximum values (12.0, 11.5 and unit/ml resp.)
240 after continues shaking the culture at 100 rpm for 96hrs with 10gm/litre of Carboxy-
241 methylcellulose as a carbon source but no xylanase activity was detected in the cell debris
242 fraction. Xylanase production has been previously reported in *Bacillus* strains [30].
243 [31] reported that all examined cellulolytic *Bacillus* species were also xylanase positive, and
244 units of xylanase activity were found to be much higher as compared to corresponding CMCase
245 activity units.
246 *P. curdlanolyticus* DSMZ-10248 appears to be xylanolytic bacteria because of its capability to
247 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 subtilis* and *Bacillus cellulosilyticus*. The pure culture of Bacillus strains: *Bacillus subtilis*-2414, *Bacillus cellulolysticus*-2579 (T) and their 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 the incubation 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.

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361