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Isolation And Identification Of Cellulose-Degrading *Lysinibacillus Fusiformis* (Accession No MG 930050) From Sawdust And Evaluation Of Its Cellulolytic Potential

Faozia Faleha Sadida
(Institute Of Education And Research (IER), University Of Chittagong, Chattogram-4331, Bangladesh

Abstract
Background: *Sawdust or wood cheeping is lignocellulosic biomass that has enormous prospects. Sawdust (having 50% carbon) releasing from saw milling is not properly processed but dumped that causes the environmental pollution. One of the most notable sources of pollution is open air burning of such wastes. Air pollution is a matter of serious concern in Bangladesh. The overall aim of this study was to produce more healthy air by converting sawdust into economically valuable products.*
Materials and Methods: *A potential cellulolytic bacteria (FFS1) was isolated from sawdust and identified based on the cultural, morphological and biochemical test as well as 16S rDNA analysis. It was studied for cellulolytic potential such as optimum condition for cellulase production and cellulase activities. Cellulase production by the bacteria was optimized using different environmental and nutritional factors such as incubation period, pH, temperature, carbon and nitrogen sources. Activity of the crude cellulase was also determined by using same factors along with metal ions, inhibitors and reductants.*
Results: *In this study a cellulolytic bacteria (FFS1) was locally isolated from sawdust and identified as Lysinibacillus fusiformis (Accession No MG 930050) based on the cultural, morphological and biochemical test as well as 16S rDNA analysis. It was evaluated for its cellulolytic potential using different physicochemical parameters. Cellulase production required pH 7.5, temperature 30^0C-40^0C whereas cellulase activity required pH 9.5, temperature 35^0C. CMC and Beef Extract were best as Carbon and nitrogen sources for both the cellulase production and activity respectively. Cellulase activity was strongly inactivated by Ag+ and Fe^{3+} ions whereas detergent SDS also inhibited the cellulase activity. As the enzyme produced by the bacteria showed highest specificity to CMC as substrate it may be an Endo-β-1,4-Glucanase. Therefore, in optimized condition the bacteria will be the important tool for future use if its cellulase is further characterized.*
Conclusion: *Locally isolated cellulolytic bacteria Lysinibacillus fusiformis have the potential for practical use for bioremediation through degradation of huge amount of sawdust.*
Keyword: *Sawdust, Lysinibacillus fusiformis, Cellulolytic Potential, Cellulase, Biomass, 16S rRNA.*

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I. Introduction

Sawdust is the main by-product or waste product of wood processing in sawmills that appears as a burden in the environment. It is a promising lignocellulosic waste composed of fine particles of wood that is generated during cutting and shredding of wood with saw and other wood processing machines [1]. Lignocelluloses has been found to be a rich source of organic components comprising cellulose, hemicellulose and lignin with a significant proportion of 45-55%, 24-40% and 18-25% respectively [2].

This waste product causes flooding, pollution etc., and destroys the aesthetic value when it is discarded into the environment. Bioconversion of the sawdust into value added products i. e. bioremediation of such burden would be a good way to overcome these problems.

The utilization of sawdust by using microorganisms for the production of industrial enzyme like cellulase will help in reducing pollution as well as valorize the waste [3-5]. Cellulases are hydrolytic enzymes that hydrolyze β-1,4-glycosidic linkages of the cellulose polymer and other related cellooligosaccharides derivatives [6-7]. Cellulase is an important enzyme for the conversion of cellulosic materials into simple sugars that serve as feed-stock for the production of different chemicals and fuels via fermentation [8]. Cellulases are employed in various important processes such as in bioprocessing industries, preparation of medicines, food production, baking, waste treatment, perfumes, textile and paper industries [3] [6]. According to global enzyme market reports, cellulase is the most demanded enzyme that covers around 20% of the global enzyme market [9].

Degradation of lignocellulosic materials in their natural environments proceeds exclusively through biological processes [10]. Several soil inhabiting microorganisms of three major groups: fungi, bacteria, and actinomycetes can produce cellulases. Cellulases of bacterial origin are more attractive because of the natural diversity and high growth rate among the major microbial groups. More importantly, cellulolytic bacteria inhabit a wide variety of environmental niches where they exhibit extremely resistant to environmental stresses [11]. Scientific research efforts on lignocellulosic wastes degradation try to improve its hydrolysis process in an economical way.

Sawdust (having 50% carbon) releasing from saw milling is not properly processed but dumped that causes the environmental pollution. One of the most notable sources of pollution is open air burning of such wastes. Air pollution is a matter of serious concern in Bangladesh. The overall aim of this study was to produce more healthy air by converting sawdust into economically valuable products. Each bacterial strain has its own identity and has to be investigated for its optimum culture conditions for its best activity for the application purpose. Moreover, high-efficient bacterial strains for hydrolyzing the sawdust and other plant wastes are not available unless paying their Know-How. Therefore, we isolated and identified an indigenous cellulolytic bacteria *Lysinibacillus fusiformis* (FFS1) from sawdust and optimized the condition for its cellulase production and cellulolytic activities.

II. Material And Methods

Isolation and Screening of the Cellulase Producing Microorganisms

Cellulase producing *Lysinibacillus fusiformis* (FFS1) was isolated from the sawdust samples collected from Hathazari, Chattogram and screened it by Congo Red Overlay Method [12]. The bacterium was isolated from the colonies showing as positive and potential cellulose-degrading ability. Then it was further screened (secondary screening) for its cellulolytic potentiality using Winstead's medium having 1.2% CMC in separate small conical flasks.

Identification of cellulolytic microorganisms

The basic routine laboratory investigation such as cultural, morphological and biochemical study which included Gram staining, Acid fast staining, Endospore staining, Indole, Methyl red, Voges-Proskauer, Citrate utilization, Catalase, Urease, Starch hydrolysis, Gelatin hydrolysis, Sugar fermentation, Caseinase, Hydrogen sulfide production and Nitrate reduction test were performed [13] and provisionally identified the isolate FFS1 as *Lysinibacillus fusiformis*. It was confirmed with the analysis of 16S rRNA gene sequence and a Phylogenetic tree was also constructed by Neighbor joining [14].

Optimization for Cellulase production

An attempt was made to optimize the culture conditions of the bacterium such as incubation period, pH, temperature, carbon and nitrogen sources requirement for maximum cellulase production. The biomass yields, extracellular protein, reducing sugar level and cellulase production of the bacterium were recorded. Effects of pH and temperature on the growth and liquefaction of cellulosic substrate were investigated and recorded. Similarly, the production of extracellular cellulase under different carbon and nitrogen sources was studied in the liquid Winstead's culture medium. Furthermore, effects of these carbon and nitrogen sources on the production of extracellular protein, reducing sugar and biomass yield were recorded. Finally, the substrate concentration was optimized using different concentrations of Carboxymethyl cellulose (CMC) based on the maximum production of cellulase, extracellular protein, reducing sugar and biomass.

Optimization for Cellulase activity

Physicochemical parameters such as different incubation time, pH, temperature, carbon and nitrogen sources on the activities of crude cellulase were investigated and recorded. To determine the cellulase activity, a reaction mixture having 2 ml filtrate + 2 ml of 1% substrate in citrate phosphate buffer + 1 ml phosphate buffer were incubated at 35°C for 2 hrs. in a water bath and the reducing sugars released was determined by Nelson's modification of Somogyi method [15]. Enzyme activity was expressed by the amount of glucose released in µg/ml of crude enzyme/hour (U/ml) [16] and soluble protein in culture filtrate was estimated following the Lowry method [17].

The filter paper containing biomass residue was dried in an oven at 80°C for a constant weight and amount of biomass was calculated by subtracting the weight of filter paper. Yield was expressed as mg/g cellulose. Effect of different metal ions as well as reductant and inhibitors on the activities of crude cellulase was investigated. Effects of nine different metal chlorides and five different inhibitors and reductants on cellulase activity were determined by adding 2 ml of culture filtrate to 2 ml of 1% CMC prepared in phosphate buffer followed by addition of 1% of metal and inhibitors and reductants solution, respectively. All tubes were

incubated for 2 hrs. in water bath at 35°C and the enzyme activity was measured according to Nelson's modification of Somogyi method [15].

III. Result

Isolation and Identification of Cellulolytic Bacteria

Twelve bacterial isolates from Sawdust samples that appeared on Czapek's Agar medium were further screened to select the potential cellulolytic ones. All the twelve isolates were screened by Congo Red Overlay Method [12] where the area of clear zone was used to select FFS1 as the best one. Gram's staining and microscopic view revealed it as a gram positive, rod shaped bacteria (**Fig. 1**). The specified biochemical tests performed on the isolate and compared with the standard description given in Bergey's Manual of Determinative Bacteriology [18] [19]. Identification was confirmed by 16S rDNA sequence analysis. Based on morphological, cultural and biochemical characteristics as well as 16S rDNA sequence the isolate FFS1 was identified as *Lysinibacillus fusiformis* (**Fig. 2**).

Optimization for Cellulase Production

Culture condition for Cellulase production by the isolate is greatly influenced by different physicochemical parameters. Therefore effects of medium pH, temperature, incubation time carbon and nitrogen sources and substrate concentration on the production of CMCase, extracellular protein, reducing sugar and biomass yield were determined. Four days of incubation period was found optimum for maximum cellulase production (data not shown).

Effect of pH

Keeping the other conditions unchanged, the enzyme activity was measured at different initial pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5 & 10.5). Maximum production of CMCase (111.99 U/ml), extracellular protein (365.41µg/ml), reducing sugar (92.28 µg/ml) and biomass yield (366.74 mg/g) by the isolate FFS1 (*Lysinibacillus fusiformis*) was recorded in culture media with pH range 6.5 - 8.5 (**Fig. 3a.b.c**). Highest cellulase production was at pH 7.5 for the isolate.

Effect of Temperature

The bacteria was cultured at different temperature (15°C, 20°C, 25°C, 30°C, 35°C, 40°C & 45°C) keeping the other conditions unchanged. Maximum production of extracellular protein (199.25µg/ml), reducing sugar (194.76µg/ml), biomass (300.06mg/g) and CMCase activity (185.02U/ml) by the isolate was recorded in culture media at temperature range 30 - 40°C (**Fig. 4a.b.c**).

Effect of Carbon Source

The bacteria was cultured at different carbon source (Avicel, CMC, Salicin, Saw dust, Rice bran & Rice straw). The highest reducing sugar (360.67µg/ml) and CMCase activity (102.62U/ml) were recorded when CMC was used as carbon source whereas highest extracellular protein (144.36µg/ml) and biomass yield (316.73mg/g) was recorded with sawdust (**Fig. 5a.b.c**).

Effect of Nitrogen source

The bacteria was cultured at different nitrogen sources (Asparagine, Ammonium sulphate, Beef extract, Peptone, Yeast extract & Urea). Maximum extracellular protein (211.28 µg/ml), reducing sugar (318.35 µg/ml) and CMCase activity (179.40U/ml) were recorded when Beef Extract was used as nitrogen source whereas highest biomass yield (440.08mg/g) was recorded with Urea (**Fig. 6a.b.c**).

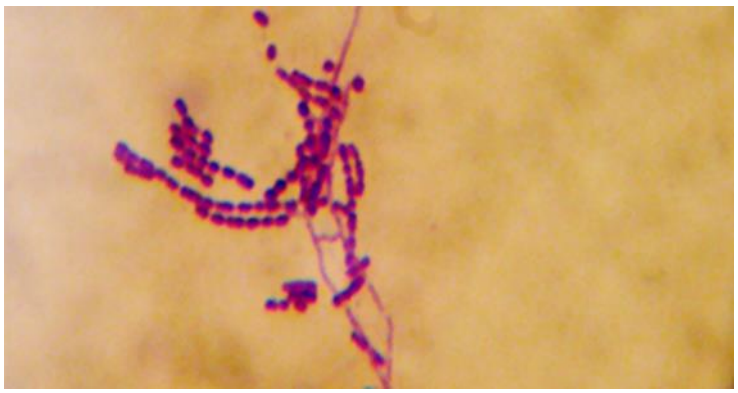

Fig 1: Microscopic view (10×100) of FSS1 (*Lysinibacillus fusiformis*)

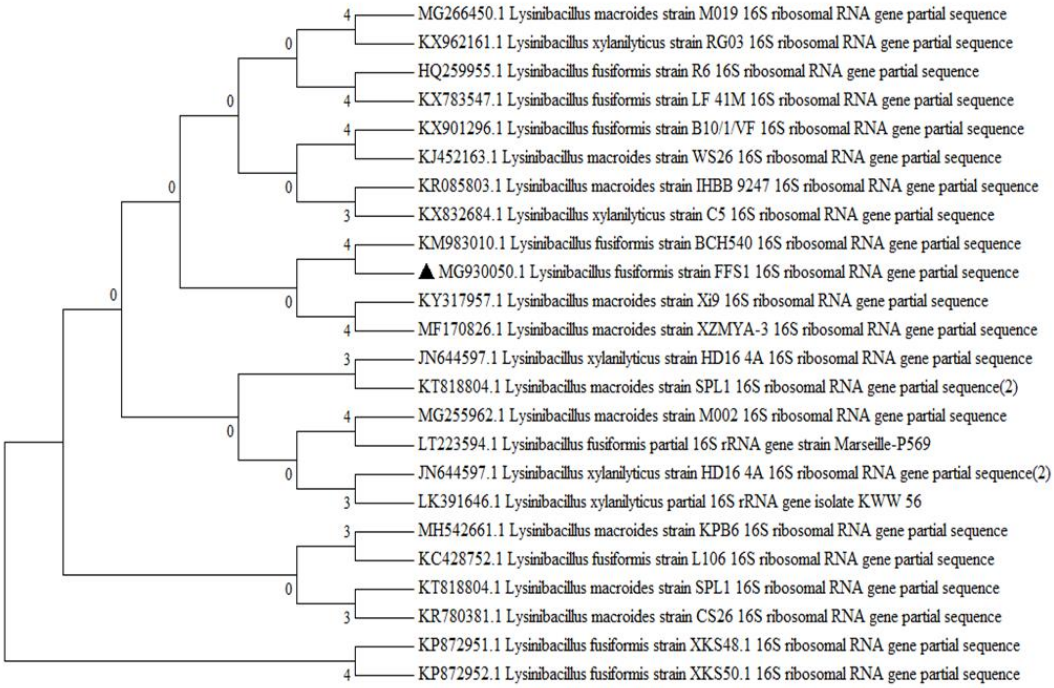

Fig. 2: Phylogenetic tree based on the 16S rRNA gene sequence of the isolate FFS1 (*Lysinibacillus fusiformis*) and related microorganisms

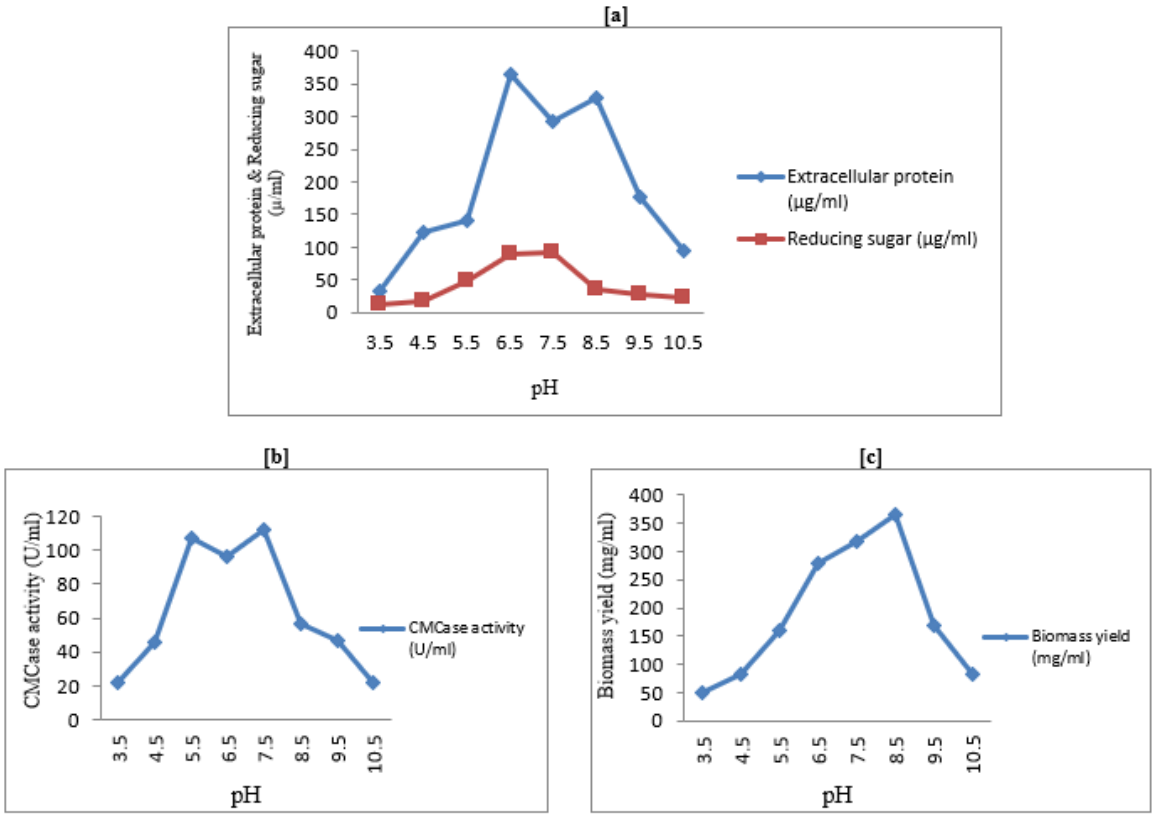

Figure 3: Effect of different pH on (a) Extracellular protein and reducing sugar production (b) CMCase activity and (c) Biomass production

Effect of Substrate concentration

The bacteria was cultured at different CMC concentration (1%, 15%, 2%, 2.5%). Maximum production of CMCase (122.10 U/ml), reducing sugar (125.09 µg/ml) and biomass (311.72 mg/g) was observed with 2.5%

(w/v) of CMC. However, the highest extracellular protein (266.92 mg/g) was recorded with 2% (w/v) CMC concentration (**Fig. 7a.b.c**).

Optimization for Enzyme activity

The quantitative cellulase (CMCase) activity of crude enzymes produced by *Lysinibacillus fusiformis* (FFS1) was investigated using different incubation time, pH, Temperature, Carbon Source, Nitrogen Sources, Metal Ions, Inhibitors and Reductants.

Enzyme-substrate reaction time

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was determined at different reaction time (30, 60, 90 and 120 min) using optimum temperature and pH, suitable carbon and nitrogen sources are shown in **Fig. 8**. Highest CMCase activity (54.68 U/ml) was recorded in 30 min of enzyme-substrate reaction time.

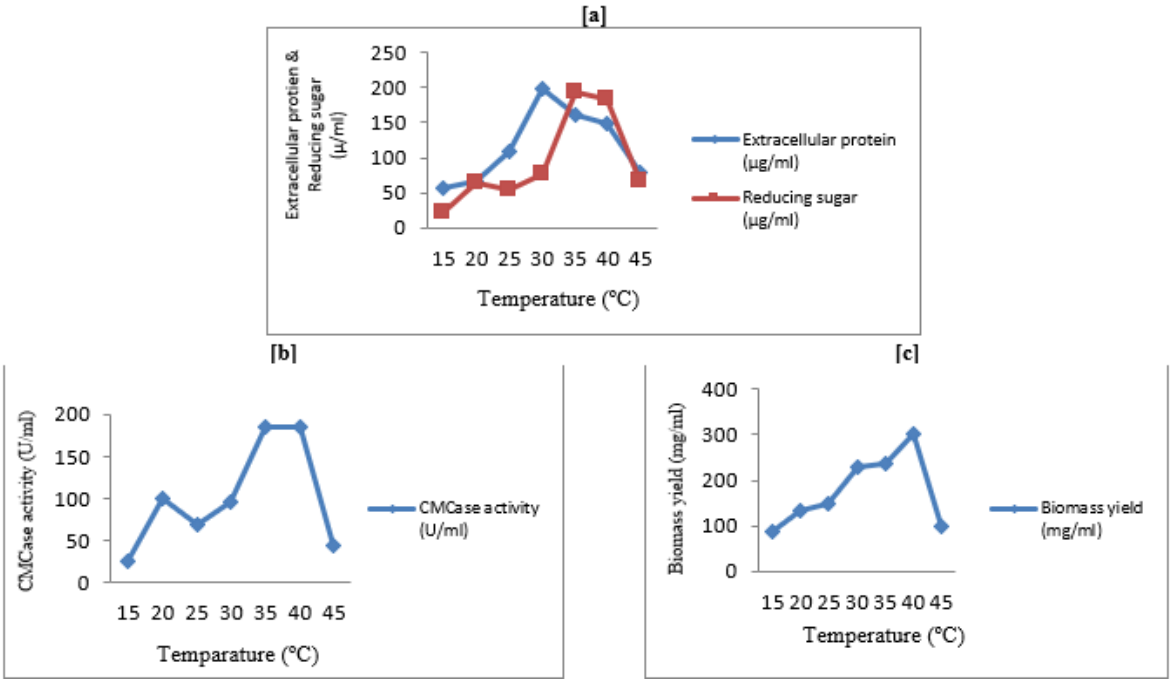

Figure 4: Effect of different temperature on (a) Extracellular protein and reducing sugar production (b) CMCase activity and (c) Biomass production

Enzyme-substrate reaction temperature

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) using suitable pH, carbon and nitrogen sources at different temperature shown in **Fig 9**. The optimum temperature during enzyme substrate reaction of crude enzyme of the bacteria was the best at 35°C (145.69 U/ml).

Enzyme-substrate reaction pH

The quantitative CMCase activity of crude enzyme of *Lysinibacillus fusiformis* (FFS1) was investigated at different pH (3.5 – 11.5) using optimum reaction time and temperature with suitable carbon and nitrogen sources are shown is shown in **Fig 10**. The highest CMCase activity by the isolate was recorded at pH 9.5 (177.99 U/ml).

Effect of Carbon Sources

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated with suitable enzyme-substrate reaction time, pH, temperature and nitrogen source in presence of different carbon sources (**Fig. 11**). The highest cellulase activity (69.29 U/ml) was recorded when CMC was used as carbon source.

Effect of Nitrogen Sources

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated with suitable enzyme-substrate reaction time, pH, temperature and carbon sources in presence of

different nitrogen sources (**Fig. 12**). The highest cellulase activity (77.53U/ml) was recorded when Beef extract was used as nitrogen source.

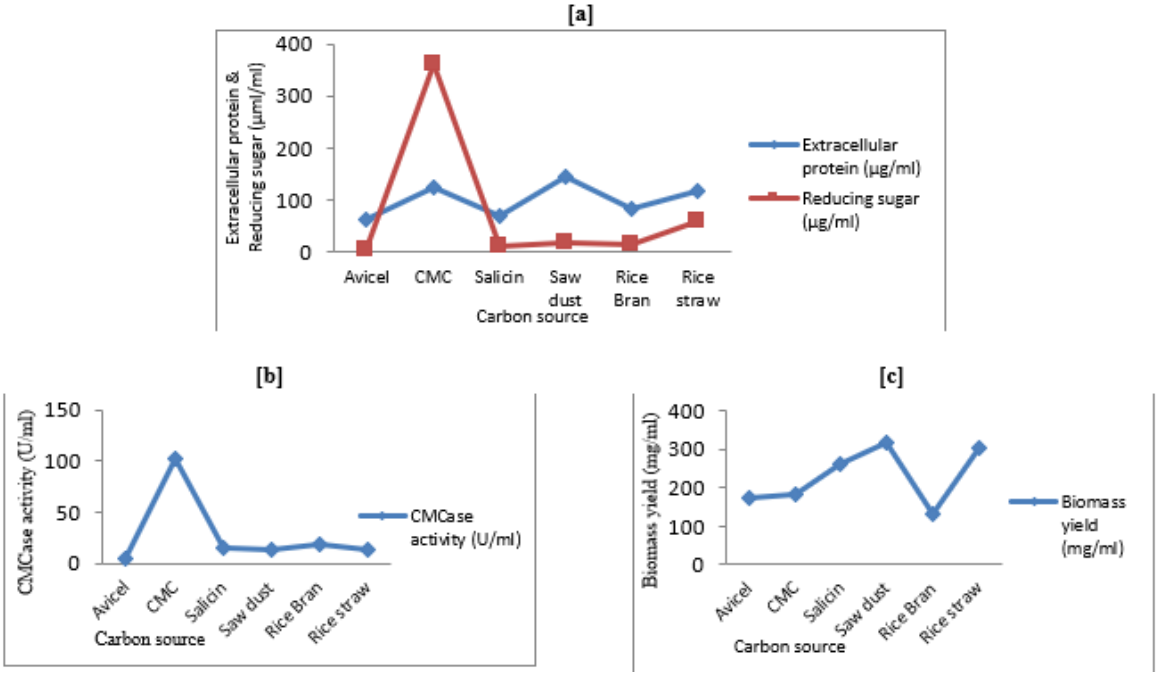

Figure 5: Effect of different carbon sources on (a) Extracellular protein and reducing sugar production (b) CMCase activity and (c) Biomass production

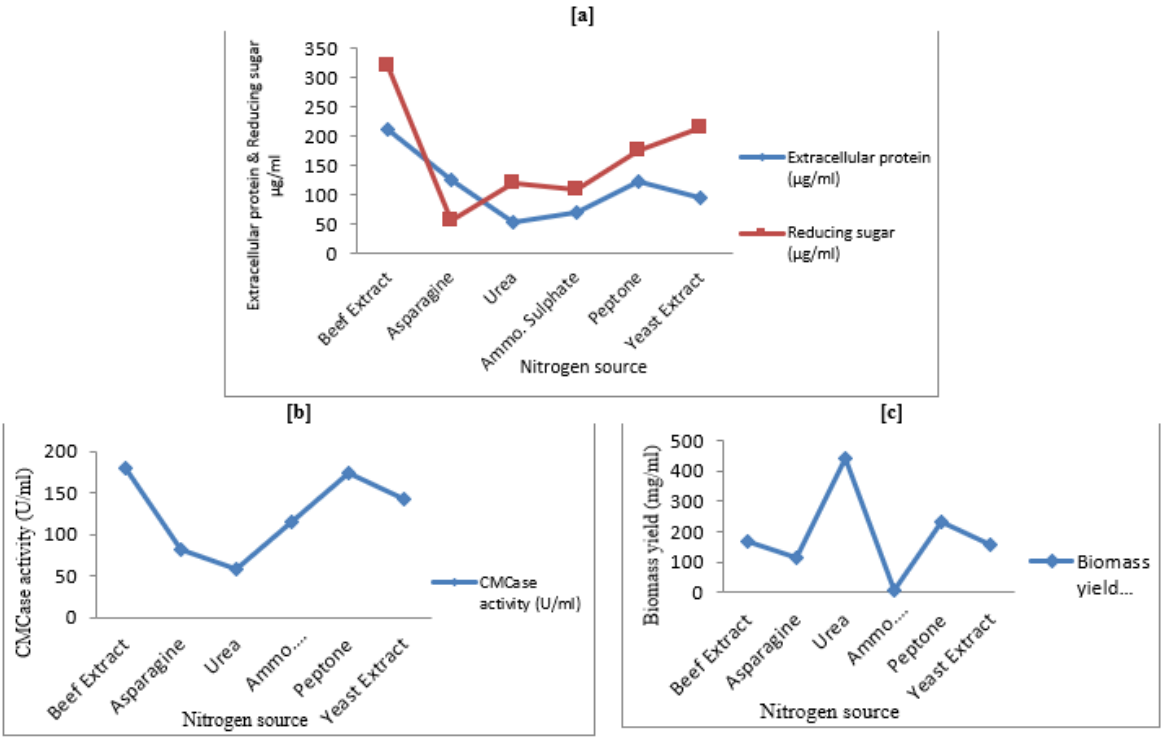

Figure 6: Effect of different nitrogen sources on (a) Extracellular protein and reducing sugar production (b) CMCase activity and (c) Biomass production

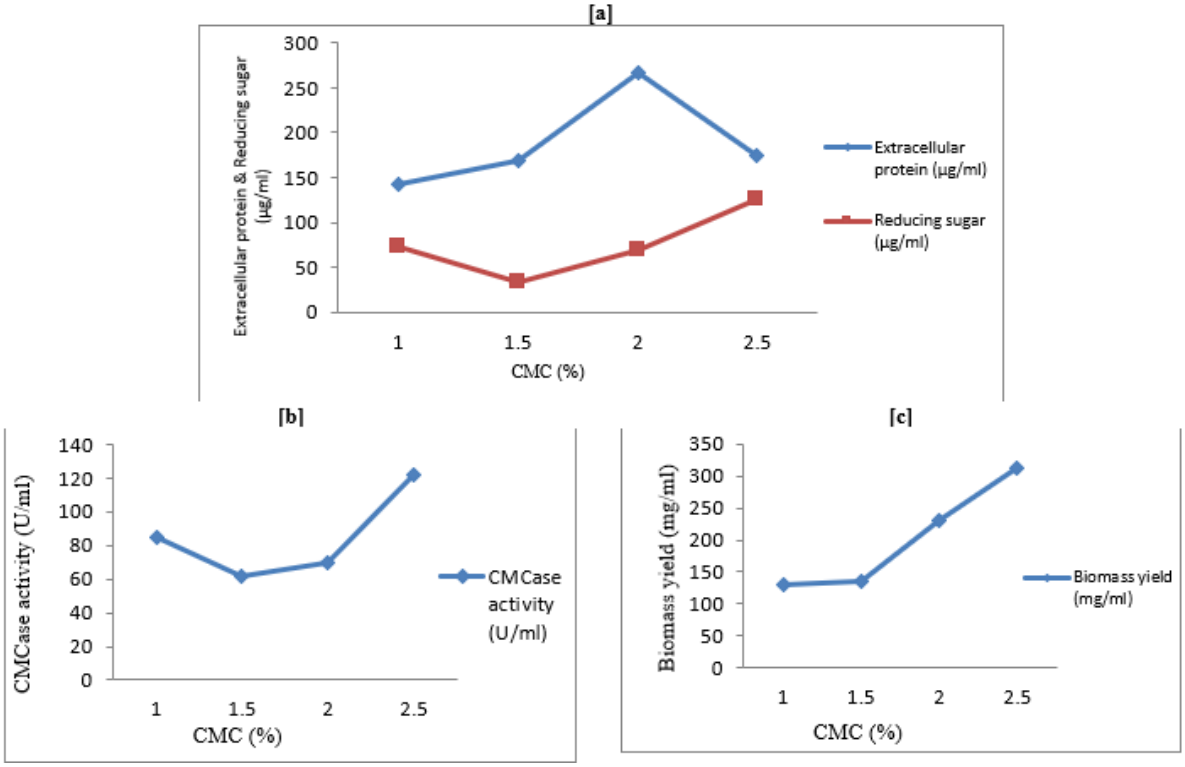

Figure 7: Effect of substrate concentration on (a) Extracellular protein and reducing sugar production (b) CMCase activity and (c) Biomass production

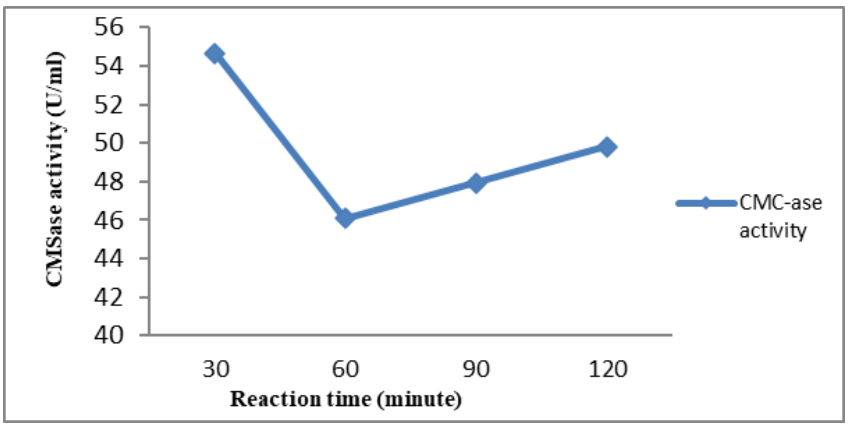

Figure 8: Relative cellulolytic activities (reducing sugar released) of crude enzymes at different enzyme-substrate reaction time

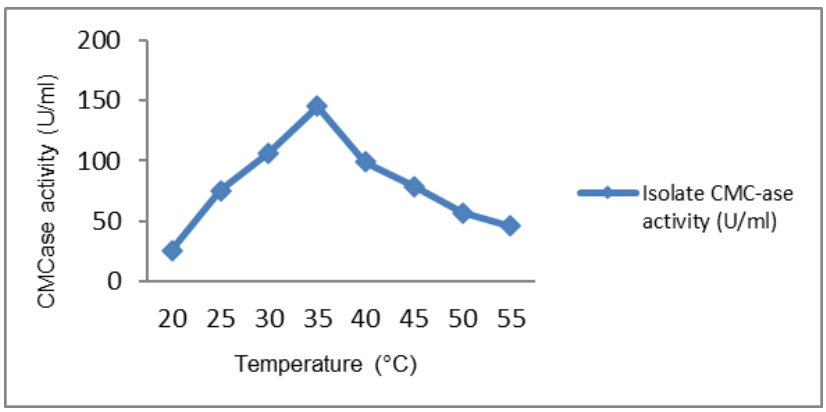

Figure 9: Relative cellulolytic activities (reducing sugar released) of crude enzymes of FFS1 at different enzyme-substrate reaction temperature

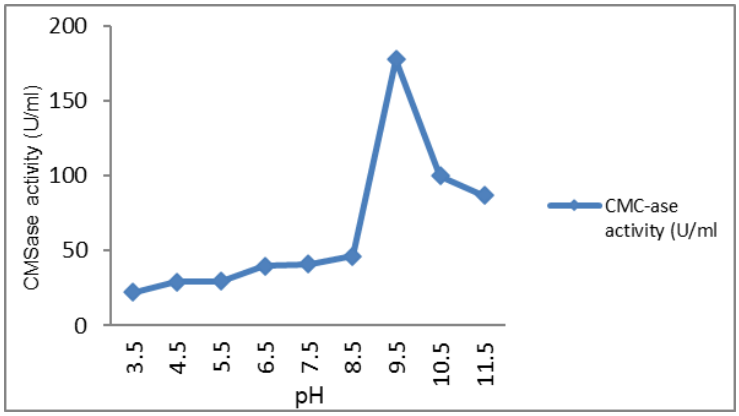

Figure 10: Relative cellulolytic activities (reducing sugar released) of crude enzymes of FFS1 at different enzyme-substrate reaction pH

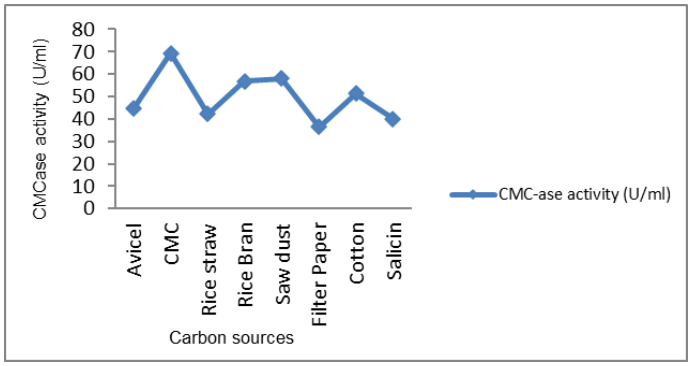

Figure 11: Relative cellulolytic activities (reducing sugar released) of crude enzymes of FFS1 in presence of different carbon sources

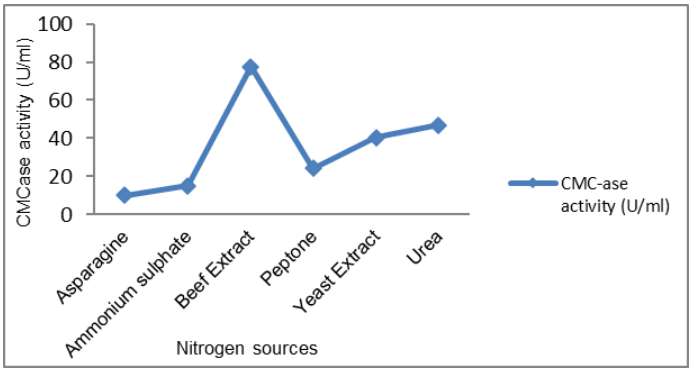

Figure 12: Relative cellulolytic activities (reducing sugar released) of crude enzymes of FFS1 in presence of different nitrogen sources.

Effect of Metal Ions

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated with suitable enzyme-substrate reaction time, pH, temperature, and carbon and nitrogen sources in presence of nine metal ions where control was maintained to compare the effects of metals (**Fig. 13**). All metal ions inactivated the CMCase activity whereas it was strongly inactivated by Ag^{+} (14.23 U/mL) and Fe^{3+} (14.61 U/mL).

Effect of Inhibitors and Reductants

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated with suitable enzyme-substrate reaction time, pH, temperature, and carbon and nitrogen sources in presence of five inhibitors and reductants where control was used to compare the results (**Fig. 14**). Almost all the chemicals showed negative results except Cysteine (105.99 U/mL) whereas SDS (26.59 U/mL) strongly inactivated the enzyme activity.

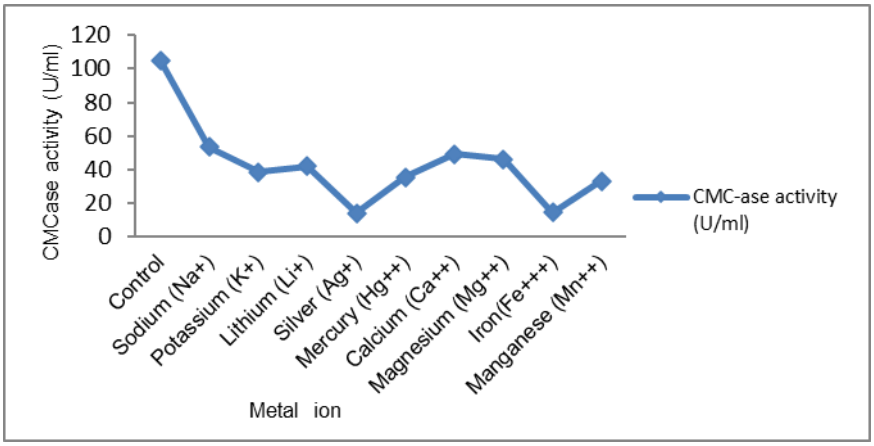

Figure 13: Relative cellulolytic activities (reducing sugar released) of crude enzymes of FFS1 in presence of different metal ions

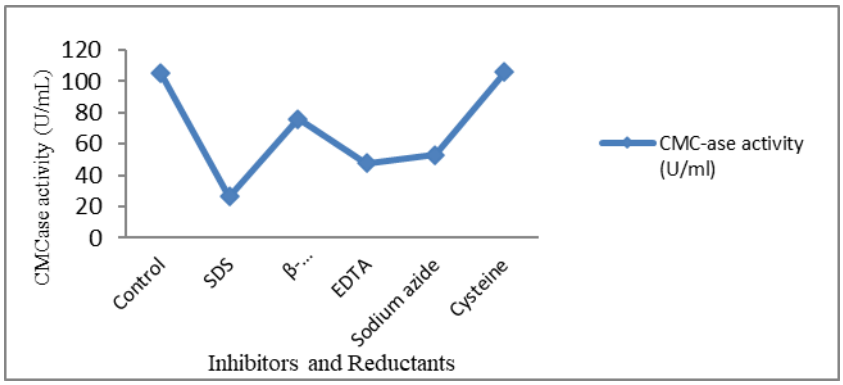

Figure 14: Relative cellulolytic activities (reducing sugar released) of crude enzymes of FFS1 in presence of different inhibitors and reductants

IV. Discussion

In this study, we investigated the cellulolytic bacteria present in sawdust of northern part of Chattogram, Bangladesh. Accordingly an attempt was made to clarify the role of isolated bacteria in the degradation of lignocellulosic materials through the characterization of enzymatic activity.

Isolation and Identification of Cellulolytic Bacteria

Although our study suggested that different bacteria are associated with the cellulolytic and hemicellulolytic activities in sawdust, FFS1 was found as dominant one. The potential cellulolytic isolate FFS1 of Sawdust was identified as *Lysinibacillus fusiformis* based on its morphological, cultural and biochemical characteristics that was confirmed with 16S rRNA sequence analysis. Because the 16S rDNA analysis the best one for accurate identification of newly isolated bacteria therefore the isolate was identified with 16S rDNA sequence analysis and submitted to the GenBank with the accession No MG 930050. Phylogenetic analysis of the 16S rDNA sequence according to the available data in NCBI indicates that FFS1 shows 99% homology with *Lysinibacillus fusiformis*.

Optimization for Cellulase production

Culture condition for cellulase production by FFS1 in different physicochemical parameters such as pH, temperature, and incubation period as well as carbon and nitrogen sources were studied as it is greatly influenced by them. The isolate produced maximum reducing sugar and extracellular protein after 4 days of incubation at 35°C and pH 7.5. Similar finding are also reported by other workers [20]. Substrate concentration on the production of cellulase was also tested using different concentrations of CMC in Winstead's broth. It showed the highest level of reducing sugar and extracellular protein production in Winstead's broth having 2%-2.5% CMC.

The effect of wide range of pH was observed during cellulase production. The maximum production of extracellular protein (162.41 µg/ml), reducing sugar (194.76 µg/ml), and biomass was recorded with pH range 6.5-8.5 that bears similarities with other workers [20-22].

The effect of temperature on cellulase production revealed that the isolate was able to produce cellulase at a range of 30°C-40°C. However, maximum production of CMCase (185.02 U/ml), extracellular protein (199.25 µg/ml) and reducing sugar (194.76 µg/ml) by the isolate was recorded in culture media at temperature 35°C, whereas highest biomass production (300.06 mg/g) was recorded at 40°C that is concurrent with the findings of other workers [23-26].

The influence of different carbohydrates: carboxymethyl cellulose (CMC), microcrystalline cellulose (Avicel), Rice straw, Rice bran and saw dust were evaluated as carbon sources for the production of cellulase by the bacteria. Highest extracellular protein (144.36 µg/ml), reducing sugar (360.67 µg/ml), CMCase activity (102.62 U/ml) were recorded when CMC was used as carbon source whereas highest biomass yield (316.73 mg/g) was recorded with saw dust. CMC gave the highest yield, followed by other carbohydrate. Similar observation was reported by other workers [20] [24] [27] [28].

Maximum extracellular protein (211.28 µg/ml), reducing sugar (318.35 µg/ml) and CMCase activity (179.40 U/ml) were recorded when Beef Extract was used as nitrogen source whereas highest biomass yield (440.08 mg/g) was recorded with Urea. Similar observation was reported by other workers [29-31].

Maximum production of CMCase (122.10 U/ml), reducing sugar (125.09 µg/ml) and biomass (311.72 mg/g) was observed with 2.5% (w/v) of CMC. However, the highest extracellular protein (266.92 mg/g) was recorded with 2% (w/v) CMC concentration. In previous studies, optimization of concentration of carbon source has been carried out by several researchers for different microbe or microbe isolated and selected from different sample. Lugani *et al*. (2015) [28] observe 1%. Akinleye *et al*. (2018) [32] found 2% CMC and Helal *et al* (2022) [30] found 2-2.5% CMC concentration.

Optimization Enzyme activity

Optimization of physicochemical factors is a very important step for successful enzymatic activity and consequently the optimum state of each factors increased the performance and better production. The quantitative cellulase (CMCase) activity of crude enzymes produced by *Lysinibacillus fusiformis* (FFS1) was investigated using different enzyme-substrate reaction time, pH, Temperature, Carbon Source, Nitrogen Sources, Metal Ions, Inhibitors and Reductants.

Enzyme-substrate reaction time

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated using suitable pH, temperature, carbon and nitrogen sources at different enzyme-substrate reaction time (30, 60, 90 & 120 min). Highest CMCase activity (54.68 U/mL) was found at 30 min reaction time. Result showed that enzyme activity is gradually decreases with the increase of reaction time [33].

Enzyme-substrate reaction pH

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated using suitable temperature, carbon and nitrogen sources at different pH (3.5 – 11.5) that showed pH 9.5 is the best (177.91 U/mL) during enzyme substrate reaction. These results are close to those found by other workers [31] [33] [34] [35].

Enzyme-substrate reaction temperature

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) using suitable pH, carbon and nitrogen sources at different temperature showed that the optimum temperature during enzyme substrate reaction of crude enzyme of the bacteria was the best at 40°C (145.69 U/ml). Similar observation with enzyme-substrate reaction temperature was reported by other workers [26] [36].

Effect of carbon sources

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated with suitable pH, temperature and nitrogen source in presence of different carbon sources. The highest cellulase activity (69.29 U/ml) was recorded when CMC was used as carbon source. This result is similar to the findings reported by other workers [20] [24] [27] [28].

Effect of nitrogen sources

The quantitative CMCase activity of crude enzyme produced by *Lysinibacillus fusiformis* (FFS1) was investigated with suitable pH, temperature and carbon sources in presence of different nitrogen sources. The highest cellulase activity (77.53 U/ml) was recorded when Beef extract was used as nitrogen source. Similar observation was reported by other workers [29-31].

Effect of metal ions

The quantitative CMCase activity of crude enzyme produced by the bacteria using nine metals comparing with a control is investigated. It was found that all the metals inactivated the enzyme activity where Silver (13.55%) and Iron (13.91%) significantly reduced it. It strongly suggests that enzyme activity i. e. degradation of cellulose is significantly influenced by metals, which is in accordance with the findings of other workers [20] [37].

Effect of inhibitors and reductants

The quantitative CMCase activity of crude enzyme produced by the bacteria using five Reductant and inhibitors comparing with a control is investigated. Almost all the chemicals inactivated the enzyme activity except Cysteine (100.92%) and strong inhibition of cellulase activity was showed by SDS (25.32%). Similar reports are available [20] [34] [37].

Present study suggested that *Lysinibacillus fusiformis* (FFS1) potentially degrades the sawdust *in vitro* optimized condition. The bacteria can be an important tool for the bioremediation of sawdust released from sawmills. Moreover, it shows the prospects for the conversion of enormous sawdust into reducing sugar for future biofuel production. Therefore, not only environmental pollution through open air burning of sawdust can be avoided but also country be benefited through using the bacteria purposively.

V. Conclusion

In the present study a potentially cellulolytic bacteria (FFS1) was locally isolated from sawdust and identified as *Lysinibacillus fusiformis* (Accession No MG 930050) based on the cultural, morphological and biochemical test as well as 16S rDNA analysis. It showed the prospective cellulolytic activity *in vitro* optimized condition. Thus it can be used for bioremediation through the degradation of huge amount of sawdust in Bangladesh. Therefore, in optimized condition the bacteria will be the important tool for future use if the cellulase it produces is further characterized. As the enzyme produced by the bacteria showed highest specificity to CMC as substrate it may be an Endo-β-1,4-Glucanase.

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