Potential Detergent Compatibility And De-staining Ability Of Cellulolytic Bacteria

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Abstract

Cellulases are inducible enzymes that are synthesized by many microorganisms during their growth on cellulosic materials. Production of cellulase enzyme from bacteria has gained interest for applications in industries owing to their stability, catalytic activity and ease of production. In current study, total 40 cellulolytic bacteria were screened by agar well diffusion assay followed by Congo red stain. Cellulolytic bacteria were evaluated for detergent compatibility and de-staining ability. Bacterial cellulase production was optimized at different environmental conditions. Biochemical testing was done following Bergey's manual. Ten cellulolytic bacteria selected, were gram positive. Bacteria showed best cellulolytic activity at 40°C to 60°C, at pH 9, lactose as carbon source and peptone as nitrogen source. Best hydrolysis zone shown was 45mm from strain MS₂2 and least zone was 6mm. The maximum detergent activity for surf excel was observed for G2 while the lowest was observed for MW₂2. Similarly, maximum activity for ariel and bonus were observed for RS5 and C3. G1 and C3 showed better clearance of ink as compared to other strains. In de-staining, G1, C1 and MW₁7 showed better clearance of edible oil and G1, C2 and MW₁8 showed better clearance of grease. These promising cellulolytic bacteria can be used for various applications in different industries.

Keywords: Cellulose, enzyme, isolation, nutrients

Introduction

Cellulose is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bio-resource produced in the biosphere (1). 4-glycosidic linkages of linear polysaccharides of glucose constitute cellulose (2). Cellulose comprises of complex structure consisting polymers like hemicelluloses and lignin forming 20 to 35% and 5 to 30% of plants respectively. Degradation of the cellulose involves hydrolysis whereas plant cell wall possesses

less number of microbes resulting in such degradation. Cellulose is highly associated with complex structural compound like starch, pectin and lignin and has secondary and tertiary configuration. These factors make it resistant to hydrolysis (3). The structure of cellulose is highly crystalline such that amorphous regions are connected to each other as well as individual chains. However, the compounds look simple chemically but the intermolecular bonding makes a complex morphology (4). The basidiomycetes are considered to be effective to degrade the cellulose and are found in abundance in dead wood or litter. The difference lies between the cellulolytic systems of fungi and bacteria in their complexity, though these do not have many taxonomic differences (5).

Cellulomonas, *Clostridium*, *Bacillus*, *Microbispora*, *Thermomonospora*, *Streptomyces*, *Ruminococcus* and *Acetivibrio* etc (5) are the examples of cellulase producing bacteria while *Fusarium*, *Chaetomium*, *Penicillium*, *Myrothecium*, *Aspergillus* and *Trichoderma* etc (2) are the examples of cellulase producing fungi. Numerous fungi such as *Trichoderma* (6), *Aspergillus* (7), *Mucor* (8), *Penicillium* (9), *Phanerochaete* and *Fomitopsis* (9) are characterized to degrade cellulose. The ability of catalysis for a single cellulase is rather low but the combined action of three cellulases is quite strong (3). When different cellulolytic enzymes act on cellulosic substrates, these give rise to synergism. But, their mechanism of synergism is yet to be discovered completely (10).

The current study involve screening and utilization of bacterial enzyme for their role in detergent compatibility and de-staining ability as cellulase enhances softness, gives brighter luminosity of colors and helps in biopolishing of fabrics while maintaining fabric integrity.

Materials and Methods

Sample Collection

A total of 5 samples including cow dung, rhizospheric soil, grapes and 2 samples from municipal waste soil were randomly collected from different areas within the premises of Multan and Bahawalpur to isolate cellulase producing bacteria. Samples were collected in sterile autoclaved bottles and stored at 4°C until delivery to the laboratory for enumeration of cellulase producing bacteria on the same day.

Isolation of Cellulase Producing Bacteria

The 0.1 mL suspension of 10^{-6} dilution in sterile 0.85% NaCl solution was transferred to Carboxymethyl Cellulose (CMC) screening medium [contain (l-1): 3.0 g NaNO_3 , 1.0 g K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KCl, 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g CMC and 15 g agar, pH 7.0] (11) and were incubated at 37°C for 2 days. The colonies were isolated and purified by streaking on CMC agar plate (12).

Primary Screening of Cellulase Activity

Bacterial isolates were individually inoculated on CMC agar plates and incubated for 2 days. The plates were flooded with 0.1% Congo red for 20 minutes and washed with 1 M NaCl for 15 minutes. The clear zone formed by the isolates was indicating their cellulase activity (13).

Secondary Screening by Well Diffusion Method

The modified agar well diffusion method employed to measure cellulase activity of crude enzyme. Sterile agar contains 1% CMC poured in sterile petri plates and after solidification agar was punched with a six millimeters diameter wells. Wells filled with 100 μL of pasteurized crude enzyme and blanks (sterile distilled water). The test was carried out in triplicate. The plates were incubated at $30 \pm 2^\circ\text{C}$ for 24 hours. After incubation, culture plates were flooded with 0.1% Congo red solution for 15 minutes and Congo red solution poured off and further plates were washed by flooding with 1 M NaCl for 10 minutes. A clear zone formation around the microbial colonies indicated the hydrolysis of cellulose or CMC. The highest cellulase activity showing colonies were selected by measuring the ratio of the colony diameter to clear zone diameter (14).

Bacterial Identification

The morphology of the bacteria as well as its biochemical characteristics provided the identification details and was identified by Bergey's Manual of Systematic Bacteriology (15).

Enzyme Production Medium

Production medium contained (g/L) glucose 0.5 g, peptone 0.75 g, FeSO_4 0.01 g, KH_2PO_4 0.5 g, and MgSO_4 0.5 g. 100 mL conical flask was filled with 50 milliliters of medium. Sterilizing the flask at 121°C for 15 minutes following by cooling, the bacterial culture was inoculated and incubated at 37°C for 24 hours. The culture was centrifuged at 5000 rpm for 15 minutes (6).

Estimation of Cellulase Activity

Cellulase enzyme activity was estimated by assay using Dinitrosalicylic acid (DNS) reagent (16) by estimation of reducing sugars released from carboxymethyl cellulose. 1% carboxymethyl cellulose of 0.5 mL was mixed with crude enzyme along with 0.5 M phosphate buffer. The incubation period was of 30 minutes at 50°C. The addition of 1.5 mL of DNS reagent led to the stopping of reaction. This was followed by boiling for 10 minutes. The absorbance was measured at 540 nm for sugars (17). The one unit (U) can be defined as the amount of enzyme activity defined in terms of quantity of enzyme released by 1 mole of glucose per minute under standard conditions (18).

Process optimization for maximum cellulase production on different parameters

pH, Temperature, Carbon Sources, Nitrogen Sources

The optimization of cellulase enzyme was checked at pH 4.0, 7.0 and 9.0 in different flasks containing broth with the help of 1 N HCl and 1 N NaOH. The incubation and inoculation of the cultures was carried out at specific temperatures. The optimization of cellulase enzyme was checked at temperature 4°C, 37°C and 60°C for 24 hours. The effect of various carbon sources such as sucrose, maltose, lactose and fructose at the concentration of 1 to 5% was examined in the production medium. Various nitrogen sources like yeast extract, peptone, urea and ammonium sulphate were examined for their effect on enzyme production by replacing 0.5% peptone in the production medium (19).

Stability with various commercial detergents

The detergent products used were ariel, bonus and surf excel. The dilution was made in double distilled water with last concentration equivalent to 7 mg mL⁻¹ in order to make imitation of washing condition. The inactivity of enzyme was carried by heating them at 100°C temperature for 15 minutes. This was followed by an adding proper concentration of enzyme to detergent solution and gestation for 1 hour at 40°C (20).

De-staining Ability

Bits of white cloth were tainted with locally obtainable permanent blue ink and the pieces were then immersed into disinfected enzyme mixed detergent solution and detergent solution without enzymes. The de-staining ability was tested after standard incubation time (10-15 minutes) at 50°C and subsequently being washed twice with water (21).

Results and Discussion

Isolation of Cellulase Producing Bacteria

A total of 40 strains were isolated from five different samples. C1, C2, C3, MS₁ 7, MS₁ 8, MS₂ 1, MS₂ 2, RS5, G1 and G2 were selected on the basis of their maximum zone of clearance ranging from 12 to 45 mm (table 1). These 10 strains were found gram positive and were further identified according to the Bergey's Manual of Systematic Bacteriology and they belong to *Bacillus sp* and *Micrococcus sp*. The production of enzyme was controlled by different parameters which can be adjusted in such a way that the maximum production can be obtained. Cellulase production appears to have a dependence on various factors including temperature, pH value, carbon and nitrogen source, detergents compatibility, and so forth (22). Ladeira, et al. (20) also indicated production of cellulase by thermophilic *Bacillus sp*.

Optimization of Cellulase Production

The maximum activity at 4°C was observed for MW₁8 while for 37°C and 60°C, the maximum activity was observed for C3 and C1 respectively. The minimum activity was observed by RS5 at 4°C and 60°C while at 37°C minimum activity was observed by G2. Similarly, for pH 4, 7 as well as 9, the maximum activity was observed for C1 and the minimum activity was observed by G1. The activity for MW₂1 in case of sucrose as the source of carbon was determined to be exceptionally higher as compared to other strains. The maximum activity was observed by MW₁8 when peptone was used as nitrogen source while the lowest activity was observed for G1 in case of ammonium sulphate as nitrogen source (fig. 1). As far as the effect of the temperature on the cellulase production is concerned; it was observed that the activity keeps on increasing till an optimum temperature. If the sample is provided with the temperature more than that optimum temperature, the enzyme undergoes disintegration resulting in lowering the cellulose production. Referring to fig. 1, it can be observed that the cellulase production for each type of strain either its source as cow dung or municipal waste or rhizospheric soil, its activity is examined to be increased

Table 1: Hydrolysis zone by cellulose producing bacterial strains

Sr. No.	Sample	Location	Origin	Strains	Zone Diameter (mm)
1	Sample 1	Poultry Farm, Multan	Cow Dung	C 1	20
2				C 2	40
3				C 3	18
4				C 4	10
5				C 5	8
6				C 6	13
7				C 7	15
8				C 8	12
9	Sample 2	Public Waste Disposal soil Ali Chowk Multan	Municipal soil Sample 1	MS ₁ 1	25
10				MS ₁ 2	36
11				MS ₁ 3	31
12				MS ₁ 3	15
13				MS ₁ 4	9
14				MS ₁ 5	6
15				MS ₁ 6	5
16				MS ₁ 7	40z
17				MS ₁ 8	38
18	Sample 3	Industrial waste soil, Bahawalpur	Municipal soil Sample 1	MS ₂ 1	36
19				MS ₂ 2	45
20				MS ₂ 3	25
21				MS ₂ 4	33
22				MS ₂ 5	39
23				MS ₂ 6	15
24				MS ₂ 7	7
25				MS ₂ 8	3
26	Sample 4	Garden Soil, The Women University, Multan	Rhizospheric Soil	RS 1	8
27				RS 2	4
28				RS 3	6
29				RS 4	11
30				RS 5	12
31				RS 6	10
32				RS 7	2
33				RS 8	5
34				RS 9	8
35				RS 10	7
36	Sample 5	Fruits Market, Kumharawala Chowk, Multan	Grapes	G 1	20
37				G 2	18
38				G 3	15
39				G 4	10
40				G 5	6

(A) Effect of temperature on cellulase producing activity

(B) Effect of pH on cellulase producing activity

(C) Effect of carbon sources on cellulase producing activity

(D) Effect of nitrogen sources on cellulase producing activity

Figure 1: Cellulase production at different environmental conditions. C1, C2, C3= *Micrococcus sp.*, MW₁ 7, MW₁ 8, MW₂ 1, MW₂ 2, RS5, G1 and G2= *Bacillus sp*

with an increase in temperature from 4°C to 60°C. It is worth noticing that for C1 strain the cellulase production dropped from 0.551 to 0.508 as the temperature increased from 4°C to 37°C. Same is the case for MW₁ 8 and G₂ strain. Thus, it demonstrated that the disintegration of enzyme causes the loss in the cellulase production with increasing temperature. Shaikh et al., 2013 shows that the pH value close to 40°C has a little effect on cellulase production. Whereas, pH 7 was considered to be most suited pH level as it resulted in maximum activity (23). For the G₂, pH varying from 4 to 6, there was 30% increase in the activity while there was a rapid increase of 70% later on. For the C1, there was an increase by 0.025 for the 50% of activity with pH varying from 4 to 7. Similarly, the same trend was observed with the decrease in the activity later on. The effect of pH on cellulase activity follows the same basic principle as that of temperature. Thus, an optimum pH corresponds to the maximum cellulase activity. In accordance to fig. 1, it can be concluded that for the strains obtained from cow dung, the optimum pH corresponding to maximum activity was achieved at pH 7. Whereas, in contrary to it, the strains obtained from municipal waste MW₁, shows the peak at pH 4. For the strains obtained from rhizospheric soil, the trend was obtained to be increasing with an increase in pH. Though, it cannot be concluded that the activity obtained at pH 9 would be its optimum pH or not. The role played by nutrient sources in production of cellulase is considered to be vital. Among the considered nutrients, carbon is regarded as primary one. Narasimha et al., 2006 study showed that by using urea as a nitrogen source and at pH 5 to be best conditions for production of cellulolytic enzymes in *Aspergillus niger*. Amongst different organic carbon sources and lignocelluloses tried in the study, carboxymethylcellulose and sawdust at 1% provided maximum yield of all 3 enzymes in *Aspergillus niger* (24). Gozan et al., 2018 showed optimum results at pH value of 6.23 and at given temperature of 40°C in cellulase producing *Bacillus sp.* (25).

Detergent Compatibility

The maximum activity for surf excel was observed for G₂ while the lowest was observed for MW₂. Similarly, the maximum activity for ariel and bonus were observed for RS₅ and C₃ respectively (fig. 2). In order to examine the detergent compatibility of the strain, the comparison can be done on the behalf of maximum cellulase production referring to be most compatible among the detergents under consideration. Referring to fig. 2, it can be observed that for the strains obtained from cow dung and municipal waste, the cellulase production was found to be touching

its peak value for the detergent to be bonus. Thus, in this case, bonus is the most compatible among these detergents. Whereas, for rhizospheric soil strains, the most compatible detergent is ariel as activity was found to be more as compare to other detergents. Ahmed, et al. (26) results showed ariel as a best detergent showing best results at 55°C following surf excel tried against protease creating *Aspergillus niger* (2). Ahmed, et al. 2016 (21) results showed that bonus and surf excel showed best detergent compatibility at 50°C and these results were similar to this study.

De-staining Ability

While comparing the results, Grapes strain 1 G₁ and Cow dung stain 3 C₃ showed better clearance of ink as compared to other strains. In de-staining of edible oil, Grapes strain 1 G₁, Cow dung strain 1 C₁ and Municipal waste sample 1 strain MW₁₇ showed better clearance of edible oil as compared to other strains and in de-staining of grease, grapes strain 1 G₁, cow dung strain 2 C₂ and municipal waste sample 1 strain MW₁₈ showed better clearance of grease and enhanced softness of cloth as compared to other strains (table 2). The incubation of enzyme at 50°C with detergent solution revealed its maximum compatibility. The cloth containing the ink, oil and grease stain was dipped into the mixed solution while one piece of ink, oil and grease stained cloth was dipped into the detergent only solution. Table 2 shows that the specific strains containing enzyme mixed detergent solution completely removed the stains present on the white cloth while the detergent only solution left the mark. In our first sample which was de-staining of ink, out of ten selected strains only grapes strain 1 G₁ and cow dung stain 3 C₃ showed better clearance of ink as compared to other strains. Other strains also showed clearance of ink but that was not as significant as the clearance observed by these two strains. In second sample which was de-staining of edible oil, grapes strain 1 G₁, Cow dung strain 1 C₁ and municipal waste sample 1 strain MW₁₇ showed better clearance of edible oil as compared to other strains. It was also observed that the addition of enzyme also improved the quality of fabric by finishing and reducing dullness, as compared with detergent solution without enzyme. This suggests that the enzyme may be useful to the detergent and laundry industries as a suitable additive to detergents for improved washing and maintenance of the fabric quality. Ahmed, et al. (21) showed that enzyme mixed with detergent solution entirely removed ink stain present on the white cloth, while with the detergent only left a spot.

Figure 2: Detergent Compatibility of Cellulolytic bacteria. C1, C2, C3 = *Micrococcus*, MW₁ 7, MW₁ 8, MW₂ 1, MW₂ 2, RS5, G1 and G2= *Bacillus*

Table 2: De-staining ability of cellulolytic bacteria

Sr. No.	Designated name	De-staining of Ink	De-staining of Edible oil	De-staining of Grease
1	<i>Micrococcus</i> -C1	++	+++	++
2	<i>Micrococcus</i> -C2	+	++	+++
3	<i>Micrococcus</i> -C3	+++	++	++
4	<i>Bacillus</i> -MS ₁ 7	++	+++	++
5	<i>Bacillus</i> -MS ₁ 8	++	+	+++
6	<i>Bacillus</i> -MS ₂ 1	++	++	+
7	<i>Bacillus</i> -MS ₂ 2	+	++	++
8	<i>Bacillus</i> -RS5	++	++	++
9	<i>Bacillus</i> -G1	+++	+++	+++
10	<i>Bacillus</i> -G2	++	+	++

Less de-staining +, Medium de-staining ++, Best de-staining +++

Conclusion

Best hydrolysis zone shown was 45 mm by *Bacillus* sp. collected from municipal soil, so potential of using microbes for production of vital enzyme like cellulase may be explored as an alternative method. It can be concluded that there was a profound influence of pH and temperature on the activity of the enzymes. Moreover, the enzyme's activity was also found to be fluctuating with respect to the nutrient sources. As far as detergent compatibility is concerned, this was found that different types of strain is compatible with different type of detergents with most of them preferably showing compatibility with bonus. It was also observed that the addition of enzyme also improved the quality of fabric by finishing and reducing dullness, as compared with detergent solution without enzyme. This suggests that the enzyme may be useful to the detergent and laundry industries as a suitable additive to detergents for improved washing and maintenance of the fabric quality.

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