

Original Research

# Production of Cellulases by *Bacillus cellulosilyticus* Using Lignocellulosic Material

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## Abstract

The enzyme cellulase, a multi enzyme complex made up of several proteins, catalyses the conversion of cellulose to glucose in an enzymatic hydrolysis. In this study biomass alkali-pretreatment followed by enzymatic hydrolysis was carried out using crude cellulase enzyme produced from screened bacillus strain (*Bacillus cellulosilyticus*) having 1,998.79 IU/ml/min CMCase and 1,621.16 IU/ml/min FPase enzyme activity. The production of cellulase enzyme using an economical medium has been a significant achievement in the field of industrial biotechnology. The maximum yield of sugars in the form of total sugars (179.84±0.2 mg/ml), reducing sugars (126.72±0.1 mg/ml), and glucose (105.40±0.1 mg/ml) was achieved at 48 h incubation time, 50°C, 5 pH, 6% enzyme concentration, 4% substrate loading, and PEG 3350 as a surfactant.

**Keywords:** *Bacillus cellulosilyticus*, Cellulases, hydrolysis, optimization

## Introduction

Cellulases are the most useful enzymes in industry. They can be produced by fungi, bacteria, or actinomycetes. The high cost of cellulases is mainly due to the substrates used in production, and also the slow growth rate of fungi. Most of the research on cellulase production has focused on fungi, with relatively lesser stress on bacteria [1]. Bacteria, which have high growth rates compared to fungi, have good potential to be used in cellulase production [2]. Also, bacteria, owing to their diversity and rapid growth, can produce both alkali-stable and temperature-stable enzymes, which can be

very important from an industrial point of view [3]. Cellulases produced by bacteria are often more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolyzed (feedback inhibition) [2]. *Bacillus cellulosilyticus*, *Alkaliphilic Bacillus* species has important industrial applications due to its ability to produce alkaline enzymes such as cellulase [4]. It produced extracellular enzymes that are resistant to high pH and high temperature conditions [4-6].

Cellulose, hemicelluloses, and lignin are major components of the lignocellulosic biomass. Cellulose binds tightly with lignin and hemicellulose. For efficient hydrolysis of cellulose, lignin components must be separated in order to make cellulose more accessible to the enzymes [7]. Prior to enzymatic hydrolysis, pretreatment is an important tool for practical cellulose

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conversion processes. Pre-treatment is required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzyme that converts the carbohydrate polymers into fermentable sugars. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose. Pretreatment also has great potential for improvement of efficiency and lowering of cost through research and development [8].

In the present research work, biomass alkali pretreatment followed by enzymatic hydrolysis was carried out using crude cellulase enzyme produced from screened bacillus strain *Bacillus cellulosilyticus* as well as to evaluate chemical composition of yard waste and to optimize enzymatic hydrolysis.

## Experimental Procedures

### Bacterial Cultures

Five cellulolytic strains of *Bacillus cellulosilyticus*, named PC-BC1, PC-BC3, PC-BC4, PC-BC6, and PC-BC8 were obtained from the culture collection center of the Food and Biotechnology Research Centre, PCSIR Labs Complex Lahore. The strains were revived on nutrient agar slants for 48 h at 30±5°C and preserved at 4°C and subcultured monthly.

### Plate Screening for Cellulase Enzyme

Pure cultures of all bacterial strains were separately transferred in CMC agar plates for plate screening by streak method and incubated at 30±5°C for 2 days to allow for the secretion of cellulase. After 2 days, the agar medium was flooded with aqueous solution of Congo red dye (1% w/v) for 15 minutes to visualize the hydrolysis zones. Then Congo red solution was poured off, and the plates were flooded with 1M NaCl solution for 15 min. To indicate the cellulase activity of the bacteria, diameters of clear zones around colonies on CMC agar were measured. The ratio of clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity. Among all *Bacillus* strains, PC-BC6 was selected as the one showing the highest cellulose degrading ability.

### Substrate Collection

Yard waste (containing Bermuda grass, tree leaves, plant stems, herbs, dry leaves) was selected as the lignocellulosic raw material. This substrate was collected from the College of Earth and Environmental Sciences (CEES), University of the Punjab. The material was sun-dried, chopped, and pulverized in a hammer bitter mill, and then sieved by maintaining 2 mm mesh size.

## Alkaline Pretreatment of Substrate

Yard waste was pretreated to remove the lignin content from substrate [9-10]. For alkaline pretreatment, 400 g of ground dry substrate was taken in a 3,000 ml flask and soaked in 4 ml of 2.5% NaOH for 2 hours at room temperature. It was then autoclaved at 121°C for 60 minutes. After that, the digested sample was recovered by filtration as the sample was filtered through muslin cloth and washed with distilled water 5-6 times to get pH 7.0. Then residues were oven dried at 60-80°C overnight, packed in polypropylene bags, and stored in desiccators until use.

## Proximate Analysis

Yard waste was subjected to proximate analysis under the standard experimental conditions according to standard method [11] for determining the percentage of moisture, ash, lignin, and cellulose content in context to investigate the chemical composition of the substrate under experimentation.

### Determining Percentage Moisture Content and Ash Test

Moisture content of yard waste was measured by simple standard method [11]. "Ash content" is a measure of the total amount of minerals present within a biomass. Ash of the pretreated and control samples were calculated according to the standard method [11].

### Lignin Test

The lignin content in treated and untreated samples was measured [12]. One gram of sample ( $W_1$ ) was taken in 250 ml round bottom digestion flask and 70 ml of 1.25% of  $H_2SO_4$  was added to reflux the sample for 2 hours. After refluxing, the sample was filtered and residues obtained on filter paper were taken in 250 ml flask and 30 ml of 72% conc.  $H_2SO_4$  was added and mixed with a magnetic stirrer for 15 minutes. Excess water was added to dilute the sample, and filtered. The residues were taken in crucible and oven dried at 105°C. The sample was weighed ( $W_2$ ), and the percentage of lignin was calculated by the formula:

$$\% \text{ age of lignin} = \frac{W_2}{W_1} \times 100$$

### Cellulose Estimation

Cellulose in the pretreated and untreated sample was estimated according to standard method [12]. One gram of sample ( $W_1$ ) was taken in a 250 ml round bottom digestion flask, and 30 ml of 80% acetic acid and 2 ml of concentrated nitric acid were added. The sample was

refluxed for 20 min and washed, filtered, and diluted with distilled water. The sample was then filtered with filter paper. Residues were taken in crucible and oven dried at 105°C overnight. The residues were weighed the next day (W<sub>2</sub>). The sample was charred for ash in a muffle furnace at 550°C for 6 hours and weighed again (W<sub>3</sub>). The percentage cellulose concentration was calculated through the formula:

$$\begin{aligned} & \text{\% age cellulose (Dry matter basis)} \\ & = \frac{W_2 - W_3}{W_1} \times 100 \end{aligned}$$

W<sub>1</sub> = Weight of material on dry basis

W<sub>2</sub> = Weight of digested material

W<sub>3</sub> = Weight of ash

### Analytical Methods

#### *Estimation of Total Sugars*

Total sugar content was measured according to the standard method [13]. For this, 1 ml of the filtrate was taken in test tube along with 1 ml of 5% phenol, mixed, and 5 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added. It was allowed for gently mixing for 20 min at room temperature (25-35°C). The absorbance of sample was taken at 470 nm against blank.

#### *Estimation of Reducing Sugars*

DNS method [14] was used to test the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose. Simultaneously 3, 5- dinitrosalicylic acid is reduced to 3-amino, 5- nitrosalicylic acid under alkaline conditions. One ml of filtrate was taken in test tube, 2 ml of distilled water, and 3 ml of DNS reagent was added and boiled in water bath for 10 min. After cooling the filtrate to room temperature, the absorbance of sample was taken at 550 nm using glucose as standard.

#### *Glucose Estimation*

The glucose was measured by using the diagnostic kit (Cat. No. 0018) PCSIR Laboratories Complex, Karachi. The kit contained reagent 1 (buffer/enzyme) and reagent 2 (standard glucose solution). The sample, standard, and blank was prepared according to the prescribed method (using kit). Readings were taken at 546 nm in a Labomed, Inc. spectrophotometer (UVS-2800) to measure the optical density (OD) of each solution. The glucose was estimated by using the following formula:

$$\text{Glucose concentration} = \frac{\text{Sample (Absorbance)}}{\text{Standard (Absorbance)}} \times 100$$

### Cellulase Enzyme Production

Three different media compositions were used for the production of cellulase enzyme using pretreated lignocellulosic material as carbon source. Among these three media m1 ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.6%, NaCl 0.5%, KH<sub>2</sub>PO<sub>4</sub> 0.3%, MgSO<sub>4</sub>·7H<sub>2</sub>O, pretreated yard waste substrate 1%), m2 (KH<sub>2</sub>PO<sub>4</sub> 0.2%, K<sub>2</sub>HPO<sub>4</sub> 0.7%, MgSO<sub>4</sub> 0.02%, NaCl 0.1%, yeast extract 0.1%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.2%, FeSO<sub>4</sub>, pretreated yard waste substrate 1%), and m3 (MgSO<sub>4</sub> 0.03%, K<sub>2</sub>HPO<sub>4</sub> 0.2%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.25%, Pepton 1%, yeast extract 0.6%, pretreated yard waste substrate 1%), which showed that maximum cellulase yield was used for further sacchrification process.

#### *Inoculum Development*

To the slants of 5-day-old cultures 1 ml of sterilized distilled water was added. The spores were dislodged to make homogeneous spore suspension. The spore count was adjusted to 5.8×10<sup>8</sup> spores/ml by Haemocytometer. One ml of this suspension was used as inoculum for 100 ml production medium.

#### *Fermentation Protocol*

Fermentation medium of 100 ml was prepared using medium composition m1, m2, and m3, then autoclaved at 121°C for 15min. After sterilization, the medium was allowed to cool at room temperature, inoculated with 1 ml of selected bacterial isolate, and incubated in a shaker (Eyela NTS- 331) at 35°C for 24 h of fermentation at 140 rpm agitation. After termination of the fermentation period the fermented broth was centrifuged at 7,000 rpm for 15 min at 4°C to remove the residual material. The clear supernatant obtained after centrifugation served as a crude enzyme source.

### Estimating Cellulase Activity

#### *Assay of CMCase*

The CMCase activity in the culture filtrate was assayed by incubating 1 ml of crude enzyme solution with 1% CMC solubilized in 0.05 M phosphate buffer (pH 7.0) at 50°C for 30 min. DNS was added in the reaction mixture boiled for 10 minutes. Reducing sugars were estimated by the DNS method [14].

#### *Assay of FPase*

Filter paper degrading activity in the centrifugal supernatant was determined by taking 1 ml of crude cellulase fraction. A Whatman No. 1 filter paper strip was immersed in 1% CMC in 0.05 M phosphate buffer, pH 7.0 [15], and 1 ml of crude cellulase. After incubation at 50°C for 60 min, the reducing sugar released will be carried out by the DNS method [14].

## Enzymatic Hydrolysis

Four grams of delignified sample (yard waste) was taken in 250 ml Erlenmeyer flasks. The cellulase enzyme (5 ml) and substrate 4 g in 0.05M citrate buffer of pH 4.8 was added and incubated at 55°C for 2-3 days. To prevent possible micro-organism contamination, 400 mg of 10 mg/ml tetracycline antibiotic in 70% ethanol and 300 mg of 10 mg/ml cyclohexamide in DI water were added to the hydrolysis broth before adding enzymes. After each day total sugars, reducing sugars, and glucose testing was performed.

### Optimizing Enzymatic Hydrolysis

The following process parameters were optimized to get a maximum of sugars from the substrate.

#### Effect of Temperature on Hydrolysis

During this work we evaluated incubation temperatures of 50, 55, 60, 65, and 70°C, keeping all other parameters constant; total sugar, reducing sugar, and glucose content were estimated.

#### Effect of Time on Hydrolysis

The incubation periods 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h were evaluated while all other parameters were kept constant; total sugar, reducing sugar, and glucose content were estimated.

#### Effect of Substrate Concentration on Hydrolysis

The substrate concentrations 2 gcm<sup>-3</sup>, 3 gcm<sup>-3</sup>, 4 gcm<sup>-3</sup>, 5 gcm<sup>-3</sup>, and 6 gcm<sup>-3</sup>, respectively, were evaluated while all other parameters were kept constant to find the total sugar, reducing sugar, and glucose contents.

#### Effect of Enzyme Concentration on Hydrolysis

The enzyme concentrations of 3%, 4%, 5%, 6%, and 7% by volume, respectively, were evaluated while all other parameters were kept constant to estimate total sugar, reducing sugar, and glucose content.

## Effect of pH on Hydrolysis

The effect of pH on cellulose hydrolysis was evaluated by conducting experiments on different pH, including 4.0, 4.5, 5.0, 5.5, and 6.0, while all other parameters were kept constant, like temperature of substrate maintained at room temperature. Diluted samples of 50 ml each were prepared and their pH was adjusted along with control to compare the effect of pH on hydrolysis.

### Effect of Surfactants

The tested additives (surfactants) were non-ionic detergent (Tween 20 and Tween 80), Polyethylene glycol (PEG 3350), SDS (Sodium dodecyl sulfate), and triton X100. The surfactant concentration was 0.1% (w/w). The conditions for the enzymatic hydrolysis with surfactants were the same as in the hydrolysis without surfactants. All experiments were performed in triplicate.

## Results

### Plate Screening for Cellulase Enzyme

All bacterial strains were streaked on CMC agar plates and incubated at 35°C for 2 days to allow for the secretion of cellulase. After washing each incubated plate with Congo red dye and 1M NaCl solution, zones of various diameters were obtained for each strain of Bacillus (Fig. 1).

The formation of zones indicated that all the bacillus strains contain cellulases, which effectively degrade CMC. The best/largest zone was formed by PC-BC6, showing 7.75 cellulolytic index, while on other hand PC-BC1, PC-BC3, PC-BC4, and PC-BC8 exhibited 4.83, 3.13, 3.47, and 5.40 cellulolytic index, respectively. So PC-BC6 was chosen for the forthcoming saccharification studies. The experiment was repeated 3 times with all strains.

### Substrate Estimation

Yard waste substrate was treated with 2.5% NaOH to de-lignify the substrate and expose the cellulose for enzyme to hydrolyze and produce the reducing sugar

Table 1. Proximate analysis of untreated and treated yard waste.

Substrate	Content			
	Lignin (%)	Cellulose (%)	Ash (%)	Moisture (%)
Untreated	18.4±0.4	46.7±0.1	2.2±0.2	5.2±0.2
Treated	5.7±0.3	79.1±0.3	4.6±0.1	7.5±0.4

± Sign indicates the S.D among three replicates

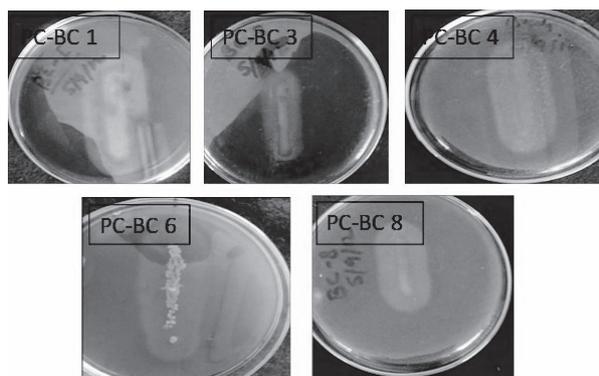


Fig. 1. Plate screening assay showing the formation of clear zones by different bacillus strains.

and glucose, which was further used in the fermentation process. The compositions of the untreated and treated substrates were determined by performing the standard method for percentage moisture, ash, lignin, and cellulose content; compositional analysis is given in Table 1. Results show that the lignin, cellulose, ash, and moisture contents of untreated substrate were  $18.4 \pm 0.4$ ,  $46.7 \pm 0.1$ ,  $2.2 \pm 0.2$ , and  $5.2 \pm 0.2$ , respectively, and for treated substrate was  $79.1 \pm 0.3$ ,  $5.7 \pm 0.3$ ,  $4.6 \pm 0.1$ , and  $7.5 \pm 0.4$ , respectively. The chemical composition of yard waste was found to vary according to its growth location, components, and the season, as well as analysis procedure.

### Cellulase Enzyme Production in Flasks

The *cellulosilyticus* strain (PC-BC6) was screened for cellulase enzyme production in the submerged fermentation process. Among the three media tested, m<sup>3</sup> revealed maximum cellulase yield, which was further used for sacchrification process. The enzyme extract obtained from filtration of the flask mixture was tested for CMCase and FPase activities, and results obtained are displayed in Fig. 2. The results revealed that the selected bacillus strain produced the highest amounts of FPase (1,621.16 IU/ml/min) and CMCase (1,998.79 IU/ml/min) for m<sup>3</sup>.

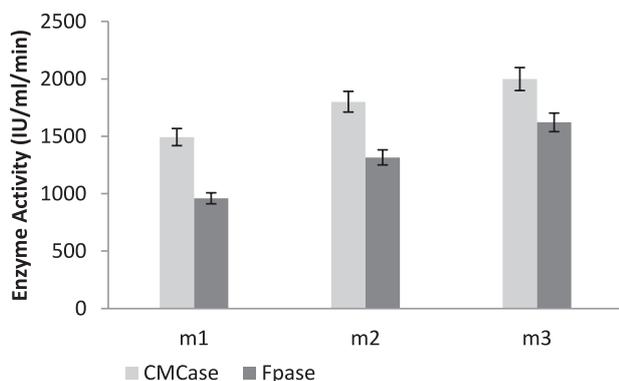


Fig. 2. Cellulolytic activity of PC-BC6 on different fermentation media.

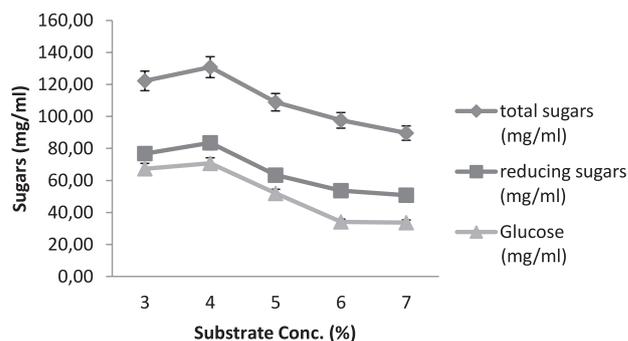


Fig. 3. Effect of various time intervals on hydrolysis of pretreated substrate.

### Enzymatic Hydrolysis

The enzymatic breakdown of treated substrate was carried out with laboratory-scale crude cellulase enzyme produced from *cellulosilyticus* under controlled fermentation to optimize various parameters.

#### Effect of Various Time Intervals on Hydrolysis of Pretreated Substrate

The hydrolysis of pretreated yard waste was performed with crude cellulase enzyme at different time periods for determining optimum time. The time effect on the enzyme was significant to the hydrolysis of the substrate as presented in Fig. 3. The results indicated that out of different time ranges, 48 h was the optimum time period for enzymatic hydrolysis of yard waste to produce the maximum sugars in the form of total sugars ( $123.03 \pm 0.3$  mg/ml), reducing sugars ( $76.82 \pm 0.4$  mg/ml), and glucose ( $67.33 \pm 0.1$  mg/ml). Further increases in the time period resulted in decreased enzymatic hydrolysis.

#### Effect of Various Substrate Concentrations on Hydrolysis of Pretreated Substrate

The suitable substrate concentration is crucial for digestion of substrate with enzyme substrate complex to produce the maximum product. The

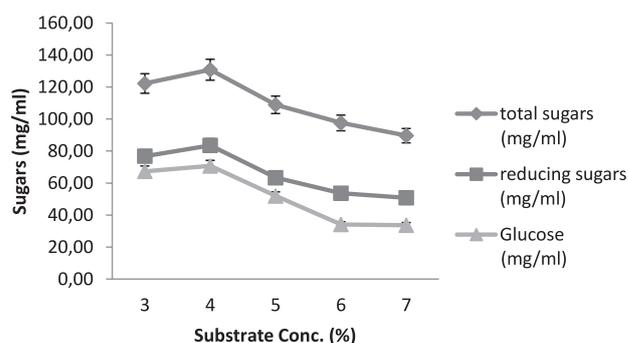


Fig. 4. Effect of various substrate concentrations on hydrolysis of pretreated substrate.

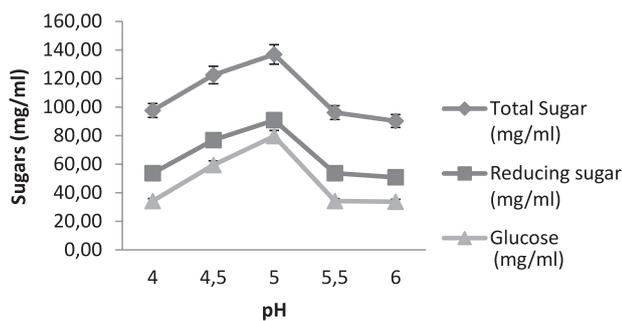


Fig. 5. Effect of pH on hydrolysis of pretreated substrate.

results presented in Fig. 4 indicated that among the various concentrations of pretreated substrate, 4% substrate concentration was found to be optimum. The substrate was found to release the maximum amounts of total sugars ( $130.80 \pm 0.1 \text{ mg/ml}$ ), reducing sugars ( $83.55 \pm 0.1 \text{ mg/ml}$ ), and glucose ( $70.68 \pm 0.4 \text{ mg/ml}$ ) at 4% substrate concentration. Further increases in substrate concentration retarded enzymatic hydrolysis.

*Effect of Various pH on Hydrolysis of Pretreated Substrate*

The enzymatic hydrolysis of substrate was performed with crude cellulase enzyme at different pH ranges for evaluating the optimum range for hydrolysis of biomass to digest the cell wall component. pH has a significant effect on the enzyme activity to induce the enzyme to catalyze the reaction (Fig. 5). The results of the study revealed that out of different pH ranges, 5 was the optimum range of pH for enzymatic hydrolysis to produce the maximum amounts of total sugars ( $136.83 \pm 0.4 \text{ mg/ml}$ ), reducing sugars ( $90.84 \pm 0.1 \text{ mg/ml}$ ), and glucose ( $79.61 \pm 0.4 \text{ mg/ml}$ ).

*Effect of Various Enzyme Concentrations on Hydrolysis of Pretreated Substrate*

The enzyme loading on the substrate was critical for proper digestion of the substrate. The enzyme was loaded at various concentrations from 3% to 7%

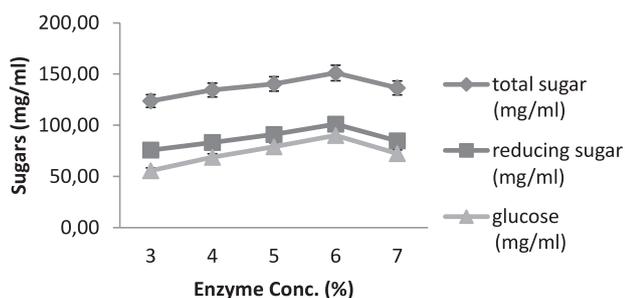


Fig. 6. Effect of various enzyme concentrations on hydrolysis of substrate.

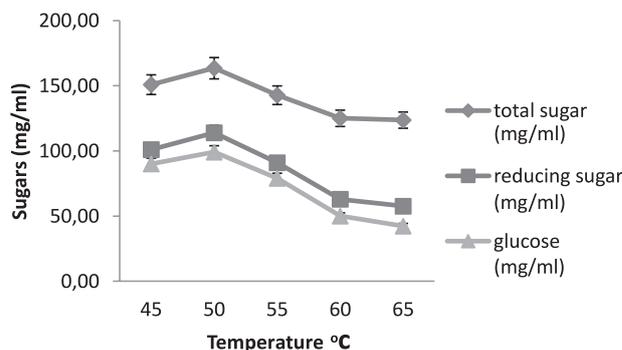


Fig. 7. Effect of temperature on hydrolysis of pretreated substrate.

(Fig. 6). The results of the enzyme loading indicated that there was progressive increase in production of total sugars, reducing sugars, and glucose up to 6% ( $1998.79 \text{ IU/ml/min}$ ) enzyme concentration per 4% substrate concentration and 6% enzyme concentration released the maximum sugars in the form of total sugars ( $151.05 \pm 0.4 \text{ mg/ml}$ ), reducing sugars ( $101.06 \pm 0.1 \text{ mg/ml}$ ), and glucose ( $90.06 \pm 0.3 \text{ mg/ml}$ ), while further increases in enzyme concentration suppressed sugar production.

*Effects of Different Temperatures on Hydrolysis of Pretreated Substrate*

The enzymatic hydrolysis of pretreated substrate was performed with crude cellulase enzyme at different temperature ranges to find the optimum range for hydrolysis of biomass. The temperature effect on enzyme activity was significant enough to induce the enzyme for full activation. The results presented in Fig. 7 depicted that out of different temperature ranges, 50°C was the optimum temperature for releasing maximum sugars in the form of total sugars ( $163.52 \pm 0.3 \text{ mg/ml}$ ), reducing sugars ( $113.96 \pm 0.1 \text{ mg/ml}$ ), and glucose ( $99.10 \pm 0.3 \text{ mg/ml}$ ). A gradual decline in sugar production was noticed by an increase in temperature (Fig. 7).

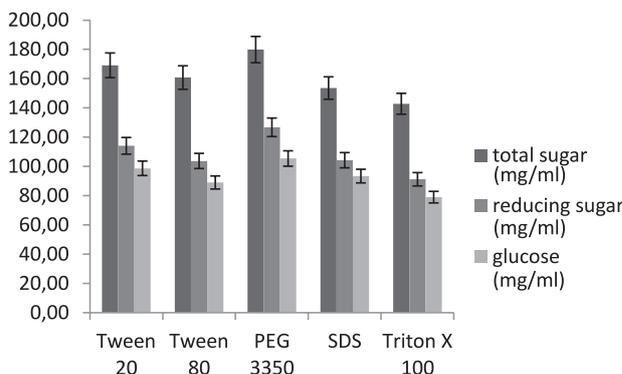


Fig. 8. Effect of various surfactants on hydrolysis of pretreated substrate.

### Effect of Various Surfactants on Hydrolysis of Pretreated Substrate

A surfactant's addition during enzymatic hydrolysis is accomplished by minimizing the irreversible binding of cellulase on cellulose and modifying the cellulose surface property. In the present study, the surfactants used in the enzymatic hydrolysis include Tween 20, Tween 80, PEG 3350, SDS, and Triton X100 (Fig. 8). It was observed from the results that the concentration of sugars improved maximum by using PEG 3350, followed by Tween 20. The maximum yield of sugars in the form of total sugars, reducing sugars, and glucose at 48 h incubation time, 50°C, 5 pH, 6% enzyme concentration, and 4% substrate concentration was 179.84±0.2 mg/ml, 126.72±0.1 mg/ml, and 105.40±0.1 mg/ml, respectively.

### Discussion

Cellulose hydrolysis is currently carried out using microbial cellulolytic enzymes. Enzymatic hydrolysis has demonstrated better results for subsequent fermentation because no degradation components of glucose are formed [16]. Cellulase research has been concentrated mostly in fungi but there is increasing interest in cellulase production by bacteria due to their higher growth rate, thermo-stable, and alkali-stable properties [17]. *Bacillus cellulosilyticus*, Alkaliphilic Bacillus species has important industrial applications due to its ability to produce alkaline enzymes such as cellulase [18]. It produces extracellular enzymes that are resistant to high pH and high temperature conditions [6, 19-21].

For the sake of efficient and rapid hydrolysis of carbohydrates the lignocellulosic material or raw material must be pretreated [9, 22]. Lignin should be removed before enzymatic hydrolysis because it acts as a protective physical barrier for cellulose to degrade in sugars and for removal of lignin alkali-pretreatment is carried out because it effectively retains most of the cellulose and removes the major portion of lignin [9, 23]. In this research work, we treated substrate with 2.5% NaOH solution to delignify the yard waste and expose the maximum cellulose for the attack of enzyme to hydrolyze the cellulose and produce sugars and glucose.

Three different media were used for the production of cellulase enzyme in this study, from which m<sup>3</sup> (MgSO<sub>4</sub> 0.03%, K<sub>2</sub>HPO<sub>4</sub> 0.2%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.25%, Pepton 1%, Yeast Extract 0.6%, carbon source, i.e., Pretreated yard waste 1%) was optimized. With m<sup>3</sup> media cellulase enzyme showed the greatest CMC<sub>ase</sub> (1998.79 IU/ml/min) and FP<sub>ase</sub> (1621.16 IU/ml/min) value. Favorable fermentation conditions and a selection of suitable growth medium played a key role in the production of cellulase [10, 24].

Factors that usually affect the enzymatic hydrolysis process of cellulose include cellulase activity, substrate

concentration, and reaction conditions like temperature, pH, and reaction time. To improve the rate and yield of saccharification or enzymatic hydrolysis, research should focus on optimizing the saccharification process [25-26]. In the present research work, among the various parameters, the loading of various enzyme concentrations during the saccharification of 2.5% NaOH-treated yard waste showed a significant effect. Cellulase enzyme loadings vary dramatically due to substrate chemical composition, pretreatment method, inhibitory compounds, and enzyme activity in the process. A study was conducted in which it was investigated that in the process of lignocelluloses saccharification with multi-enzyme their hydrolytic efficiency depends on the individual properties of enzyme [27-28] and multi-enzyme ratio [29-30]. Substrate concentration is one of the main factors that affect the initial rate of enzymatic saccharification of cellulose and an increase of substrate concentration normally results in an increase of reaction rate of the hydrolysis [31-32]. However, high substrate concentration, which substantially lowers the rate of the hydrolysis, can cause its inhibition [33-36].

The result of the temperature optimization indicated that out of different temperature ranges, 50°C was the optimum temperature range for saccharification of treated substrate with cellulase enzyme for the release of maximum sugars. Maximum hydrolysis of alkali-treated substrates occurred at 50°C, which counted the degree of saccharification of 37.29% and decreased at 60°C, which counted to a degree of saccharification of 33.11% [37].

Results predicted that out of different pH ranges, pH 5 was the optimum for enzymatic saccharification of alkali-pretreated substrate in the form of total sugars (136.83±0.4 mg/ml), reducing sugars (90.84±0.1 mg/ml), and glucose (79.61±0.4 mg/ml). It was reported in a similar study that enzymatic hydrolysis carried out at 50°C and pH 4.8 for 96 h and 58% yield was obtained, and 14.4% glucose for corn and stover, 13.1% glucose for straw of wheat [38-39]. In a parallel study [40-41], evaluated enzymatic hydrolysis of wheat straw for the conversion of cellulose and hemicelluloses into maximum monomeric sugars and reported that maximum yield of monomeric sugars (565±10 mg/g) was obtained at 45°C temperature and pH 5.0 for 72 h by using cellulase.

Reaction time is also related with the process scale-up. The fermentable sugars obtained from the hydrolysis process could be fermented into ethanol. The results were indicated that out of different time ranges 48 hours was the optimum time period for enzymatic hydrolysis of treated substrate (yard waste) to produce the monomeric sugars in the form of total sugars (123.03±0.3 mg/ml), reducing sugars (76.82±0.4 mg/ml), and glucose (67.33±0.1mg/ml) (Fig. 2). It was reported that cellulosic biomass was highly digestible after the pretreatment with yield of 90%, less than 5 days and 3 days, preferably with 10 fpu/gm cellulose [42-43].

Adding surfactant during enzymatic hydrolysis is accomplished by minimizing the irreversible binding of cellulase on cellulose and modifying the cellulose surface property. In the present study, the concentration of sugars improved the maximum by using PEG 3350, and then Tween 20 showed the highest peak. Recent studies show that enzymatic hydrolysis yield can be enhanced by using surfactants (Tween-20, Tween-80, PEG, or BSA) [33-35]. It has also been published that various surfactants are able to increase the stability of cellulase [44].

### Conclusion

Crude enzyme was produced from *Bacillus cellulosityticus*. Optimized enzymatic hydrolysis was obtained at 6% (v/v) enzyme concentration, 50°C, 4% (w/v) substrate concentration, 48 h incubation time, pH 5.0, and PEG 3350 as surfactant for enhancing the glucose concentration. The maximum yield of sugars in the form of total sugars, reducing sugars, and glucose after optimizing the hydrolysis process was 179.84±0.2 mg/ml, 126.72±0.1 mg/ml, and 105.40±0.1 mg/ml. Further study on this topic is still required to make the process economical on a commercial scale in Pakistan.

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