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Article

# Production of Cellulases by *Aspergillus unguis* and Enzymatic Hydrolysis of Alkali-treated Groundnut Fodder Using Solid State Fermentation

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**ABSTRACT**

Groundnut fodder was used as substrate for cellulase production by solid state fermentation by *Aspergillus unguis*. Different concentrations of NaOH on cellulase production and effect of temperature, pH, and surfactants were optimized to enhance cellulase production and reducing sugars yield, respectively. Under the optimized conditions, 1% NaOH-treated groundnut fodder produced maximum filter paperase activity with 5.45 FPU/g of substrate, carboxymethyl cellulase activity of 4.75 U/g of substrate, and β-glucosidase activity of 19.0 U/g of substrate at 1 ml of crude cellulase preparation. Higher yield of reducing sugars was obtained at 50°C with 4.8 g/l, pH 4.8 with 4.37 g/l. Non-ionic surfactant Tween-80 increased the rate of reducing sugars yield at all temperatures such as 40°C, 45°C, and 50°C. pH 4.8 with addition of Tween-80 showed improvement in reducing sugar yield about 2-fold by 7.16 g/L.

**Key words:** *Aspergillus unguis*, Surfactants, Alkali pretreatment, Hydrolysis, Cellulase, Reducing sugar.

## 1. INTRODUCTION

Lignocellulosic biomass is the most abundant organic material on earth and could provide inexpensive, renewable, widely available and environment-friendly feedstock for the production of bioethanol, and other value added chemicals in near future [1]. The general composition of this lignocellulosic biomass consists of cellulose (35–50%), hemicellulose (20–35%), lignin (15–25%), and a number of other compounds make up the residues [2]. Thus, cellulose being the most abundant polysaccharide constituents of cellulosic biomass and mostly found in crystalline, water-insoluble form [3,4]. Nowadays, cellulose is an attractive source of fermentable sugar, the glucose, which can be obtained by enzymatic hydrolysis of the cellulose in a process known as saccharification [5]. However, the native cellulose is buried in a matrix of hemicellulose and lignin posing physical barrier to its accessibility. Lignin present as a cover makes the entire structure recalcitrant [6]. Therefore, a pretreatment must be applied to remove lignin, decrease cellulose crystallinity, and increase the surface area for enzymatic activity [7]. Various physical, chemical, and biological pretreatment methods have been investigated by various researchers in the last three decades. Among all the investigated pretreatment methods, chemical pretreatment has been proven to be a promising one. Alkaline pretreatment is one of the extensively studied chemical pretreatment methods, which employs various alkali compounds such as sodium hydroxide [8], calcium hydroxide (lime) [9], potassium hydroxide [10], aqueous ammonia [11], ammonium hydroxide [12], and hydrogen peroxide or combination of these [13].

Cellulases are the key enzymes in enzymatic saccharification of the cellulosic biomass [14]. The complete cellulase system is comprised endoglucanases, exoglucanases, and β-glucosidases, which act synergistically for complete hydrolysis of cellulose to sugars [15]. A wide variety of microorganisms, including bacteria, fungi, and actinomycetes, are known to produce cellulases [16]. Direct use of enzymatic preparations for the simplification and bioconversion of lignocelluloses is an innovative approach [17]. However, there is no much information is available on the degradation of lignocelluloses directly using crude enzyme extracts. The efficient and reproducible bioconversion of the agricultural lignocellulosic residues on an industrial scale using enzymatic preparation may have many practical applications for the hydrolysis of lignocellulosics [18]. Hence, the influence of various factors affecting cellulases production and enzymatic hydrolysis of alkali-treated groundnut fodder such as temperature, pH, and surfactants were studied to obtain maximum yield of reducing sugars. The main objective of the present study was the utilization of inexpensive and abundantly available lignocellulosic biomass for cellulase production using crude enzyme preparations in solid state fermentation (SSF) by *Aspergillus unguis*.

## 2. EXPERIMENTAL

### *2.1. Substrate Collection*

Groundnut fodder was chosen as solid matrices for use in SSF in this study because of its abundance in the local area at cheaper rates. Coverage of the huge extensive area of cultivable land with groundnut crop in a single district of Anantapur, Andhra Pradesh, India, generates high volumes of groundnut vegetative biomass. Groundnut fodder was collected from local mills in Kadapa, Andhra Pradesh, India, and air dried. The substrate was grind and sieved through a 2 mm screen, for uniform particle size and used for subsequent experiments.

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### 2.2. Microorganism

An indigenous culture of *A. unguis* (Accession number KX816008) isolated from soil sample, collected from Kadapa Cotton Ginning Mills and first reported fungi from our laboratory [19]. Pure culture was grown on Czapek Dox agar medium at 30 ± 2°C and maintained at 4°C. Spore suspension was prepared by harvesting the spores from 7 days grown old slants by adding adequate amount of sterile distilled water with Tween-20 (0.01%).

### 2.3. Modification of Groundnut Fodder

Modified groundnut fodder was used in this study. The following method was used for modification of the substrate. Groundnut fodder was mixed with 1%, 2%, and 3% (v/v) NaOH solutions at a solid-to-liquid ratio of 1:10 (w/v), autoclaved at 121°C for 30 min. The autoclaved groundnut fodder was rinsed with distilled water till it gets neutral pH. Collected biomass was dried in an oven at 40°C until the weight was constant.

### 2.4. SSF

SSF was carried out in 250 ml Erlenmeyer flasks. 10 g of crude and modified ground fodder was dispensed. Each flask was covered with hydrophobic cotton and autoclaved at 121°C for 15 min. Sterile solid culture medium in the flasks was inoculated with the spores of *A. unguis* at density of $2 \times 10^5$ spores/flask and incubated at ambient temperature (30 ± 2°C) for up to 5 days. At the regular intervals, the samples were withdrawn for processing. Entire fermented substrate in the flask was mixed with distilled water, the slurry was filtered through muslin cloth and the filtrate was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was used for the determination of enzyme activity [20].

### 2.5. Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out with 10 g (dry weight) groundnut fodder at 50°C in 250 ml Erlenmeyer flasks with a total volume of 100 ml (0.05 M citrate buffer, pH 4.8) using crude cellulase preparations. The system was supplemented with 0.005% sodium azide to prevent microbial growth. The flasks were agitated at 150 rpm in orbital shaker at 50°C for 5 days. The influence of temperature (40°C, 45°C, and 50°C), pH (3.0–6.0), and three different nonionic surfactants (1.0% v/v), namely, Tween-20, Tween-80, Triton X-100, and ionic surfactants sodium dodecyl sulfate (SDS) on enzymatic hydrolysis was studied. Samples were withdrawn every 24 h, centrifuged at 10,000 g for 15 min, and the supernatant was analyzed for total reducing sugars and analyzed by dinitrosalicylic method.

### 2.6. Enzyme Assays

Each sample was monitored for filter paperase (FPase), carboxymethyl cellulase (CMCase), and β-glucosidase activity. Filter paper assay method [21] was employed to measure total cellulase activity of *A. unguis* grown on SSF. Activity of cellulase was expressed in filter paper units. One unit of FPase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per minute. Activity of endoglucanase in the culture filtrate was quantified by CMC method [22]. One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per minute. β-glucosidase activity in the culture filtrate of *A. unguis* was determined according to the method by Herr [23]. Activities of FPase, CMCase, and β-glucosidase were measured on substrate – filter paper, CMC, and *P*-nitrophenyl β-D-glucopyranoside, respectively, with appropriate control.

### 2.7. Protein Determination

Aliquots of *A. unguis* culture filtrate with appropriate dilution were used for the estimation of protein content according to the method of Lowry *et al.* [24].

### 2.8. Estimation of Reducing Sugars

The reducing sugar was determined by the dinitrosalicylic acid method as described by Miller [25] using glucose as the standard.

### 2.9. Statistical Analysis

Data presented is the averages of three replicates. Duncan's Multiple Range test for all data was carried out by Megharaj [26].

## 3. RESULTS AND DISCUSSION

### 3.1. Pretreatment of Groundnut Fodder by Dilute Sodium Hydroxide

Effect of pretreatment of sodium hydroxide on groundnut fodder was evaluated to optimize the sodium hydroxide concentration for effective treatment for using groundnut fodder as a component of the medium further to increase cellulase production by *A. unguis*. Effect of three different concentrations of NaOH, i.e., 1%, 2%, and 3% was studied on the production of cellulolytic enzymes. The results presented in Figure 1 indicate that FPase production by *A. unguis* was negligible on first 2 days of incubation from all the three concentrations of NaOH pretreated groundnut fodder. Later, it reaches its maximum titer (5.45 FPU/g of groundnut fodder) on 1% NaOH-treated substrate on the 3rd day of incubation and further decreased the titer of FPase with increasing the incubation period.

*A. unguis* was grown on groundnut fodder which was pretreated with different concentrations of NaOH to optimize the NaOH concentration for effective pretreatment for enhanced CMCase production (Figure 2). It was found that the highest activity of CMCase was obtained as 4.85 U/g of groundnut fodder from 2% NaOH pretreated substrate on the 1st day of incubation and gradual decrease was observed with increasing the incubation period.

Unlike other two enzymes, the β-glucosidase production was gradually increased from the 1st day of incubation from 1% NaOH pretreated substrate and reaches its maximum production with 19 U/g of groundnut fodder (Figure 3). Less β-glucosidase secretion was observed from 3% NaOH pretreated substrate on the 5th day of incubation.

Secretion of extracellular protein was increased with increasing the pretreatment concentration of NaOH. Growth of *A. unguis* on 3% NaOH pretreated groundnut fodder secreted maximum protein content with 62 mg/g of groundnut fodder on the 2nd day of incubation, whereas least secretion was recorded as 29 mg/g of groundnut fodder with same concentration on the 3rd day of incubation (Figure 4).

Alkali pretreatment is one of the most effective pretreatment methods for breaking the ester bonds between lignin, hemicellulose and cellulose, and avoids fragmentation of the hemicellulose polymers. It also causes swelling of treated substrate through the reactions salvation and saponification, which leading to decrease in the degree of polymerization and crystallinity, increase in internal surface area, disruption of the lignin structure and breaking of the structural linkages between lignin and carbohydrates [27]. According to Kim and Holtzappl [28], an effective pretreatment process should remove all of the acetyl groups and reduce the lignin content to about 10% in the treated biomass, which intern increase the accessibility of hemicelluloses and cellulose to hydrolytic enzymes [29]. Previous studies reported that the cellulose conversion improved with increasing lignin removal [30]. Lignin can absorb protein from aqueous solutions, and then, lignin removal should improve the hydrolysis of cellulose by reducing non-specific adsorption of cellulase enzymes [31]. The main advantages of alkali pretreatment method were reported to cause less sugar degradation than acid pretreatment and exhibit lesser hemicellulose and cellulose loss than acid or hydrothermal processes [32]. The pretreatment with 1% NaOH was the most effective one on increasing the cellulase enzyme production compared with other two pretreatment methods.

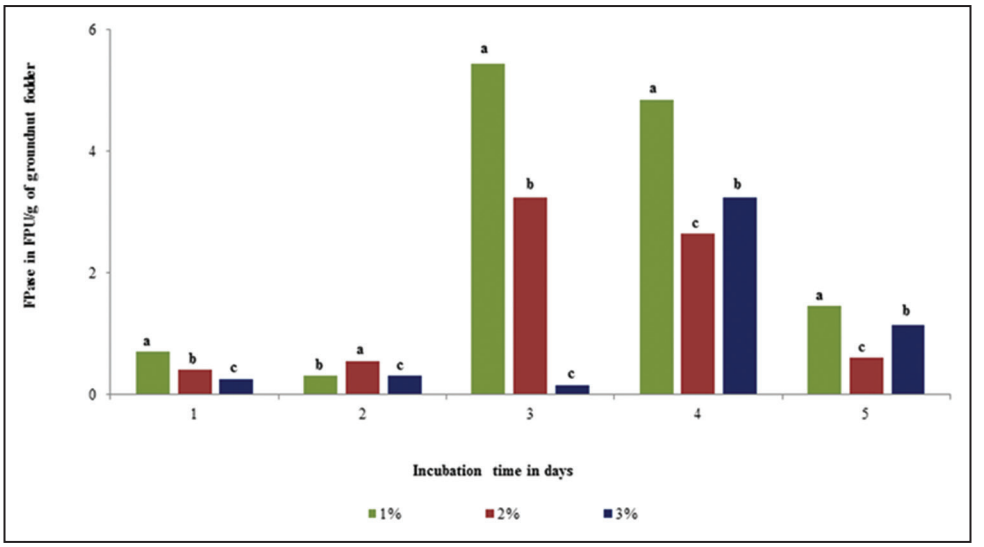


**Figure 1:** Effect of pretreatment with different concentrations of NaOH on biosynthesis of FPase by *A. unguis* under solid state fermentation.

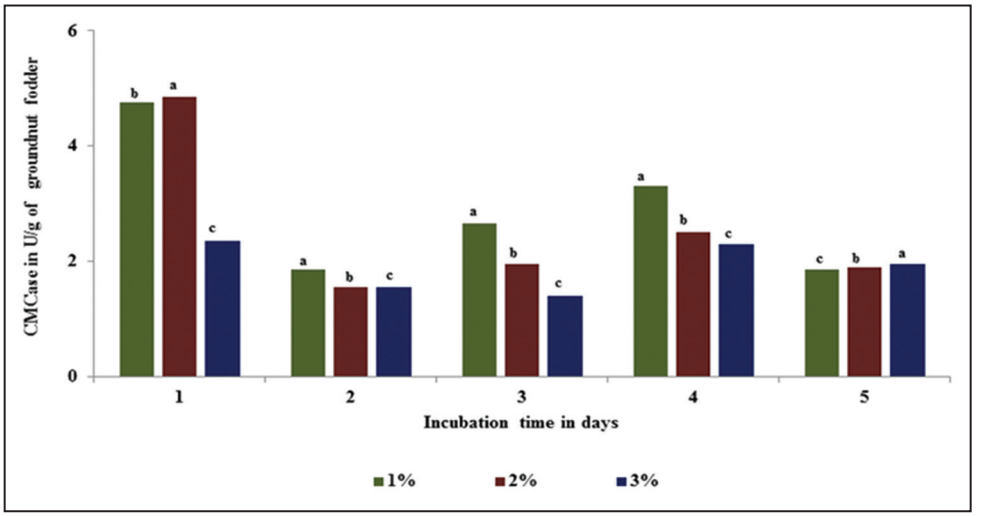


**Figure 2:** Effect of pretreatment with different concentrations of NaOH on biosynthesis of CMCase by *A. unguis* under solid state fermentation.

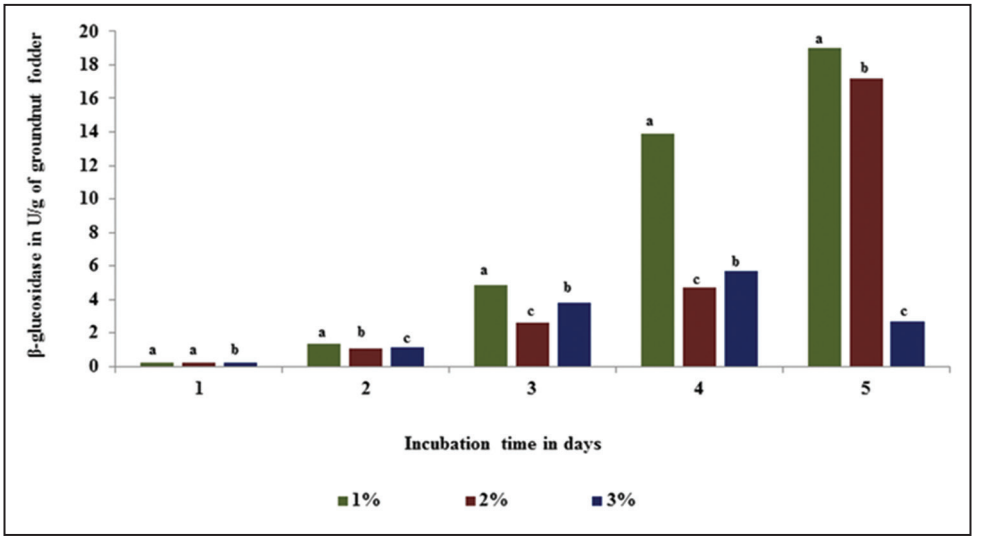


**Figure 3:** Effect of pretreatment with different concentrations of NaOH on biosynthesis of β-glucosidase by *A. unguis* under solid state fermentation.

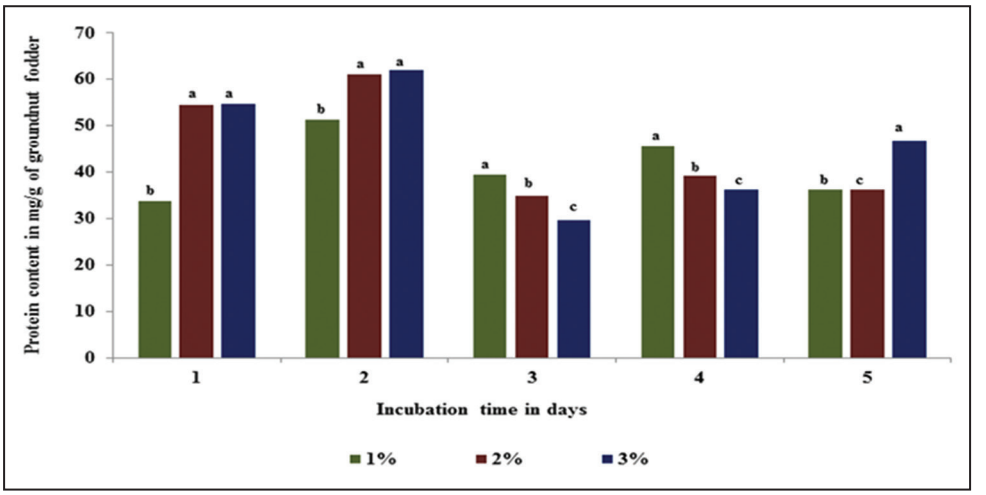


**Figure 4:** Effect of pretreatment with different concentrations of NaOH on secretion of proteins by *A. unguis* under solid state fermentation.

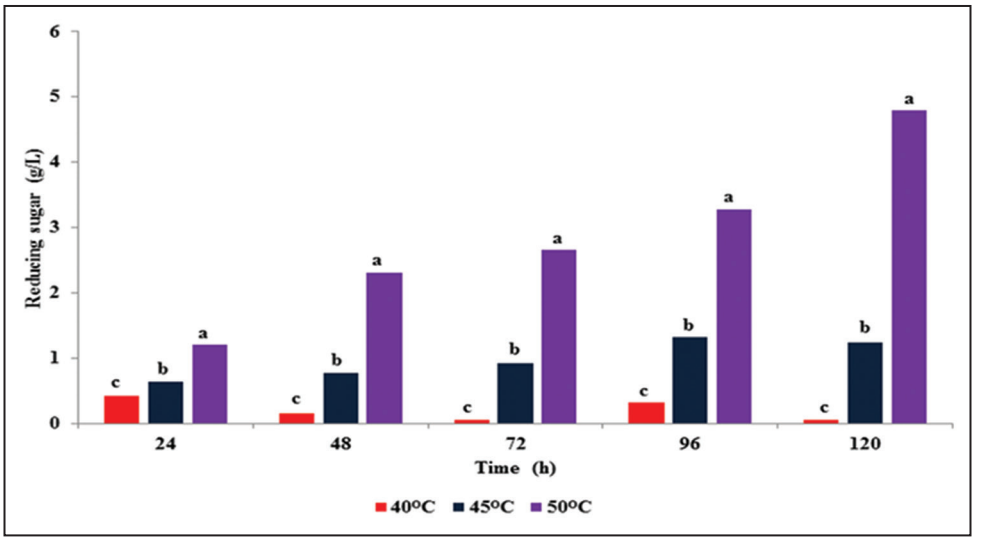


**Figure 5:** Effect of different temperatures on enzymatic hydrolysis of groundnut fodder.

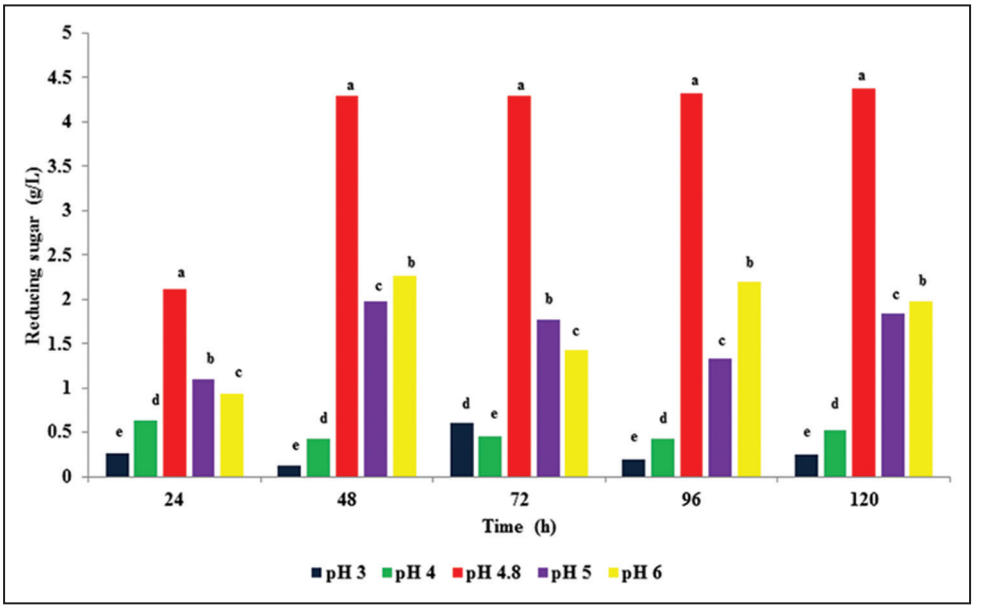


**Figure 6:** Effect of pH on enzymatic hydrolysis of groundnut fodder.

### *3.2. Effect of Temperature on Enzymatic Hydrolysis*

At 40°C, 45°C, and 50°C, the reducing sugar yields were 0.43 g/l, 1.33 g/l, and 4.8 g/l, respectively. Maximum rate of hydrolysis was achieved at 50°C (Figure 5).

### *3.3. Effect of pH on Enzymatic Hydrolysis*

Maximum amount of reducing sugar released was 4.37 g/l at pH 4.8 (Figure 6). These results are consistent with few earlier reports such as Singh *et al.* [33] and Krishna and Chowdary [34]. They found that pH has a significant effect on the hydrolytic behavior of cellulases; the hydrolytic reaction is possible only after enzyme–substrate complex formation, and the effect of pH on the both adsorption and hydrolysis is similar that usually occur at around pH 4.8. Moreover, Mahamud and Gomes [35] reported maximum hydrolysis of alkali-treated sugarcane bagasse at pH 5.0. Furthermore, Baig *et al.* [36] reported that pH 6.0 was optimal for enzymatic saccharification of steam-treated leaves and pseudostem of banana. Conversely, Karmakar and Ray [37] reported pH 7.0 as optimal for saccharification of various agro-wastes, such as orange peel, sugarcane bagasse, dried flower, water hyacinth, and coconut shell, while using cellulase enzymes from *Rhizopus oryzae* PR 7.

### *3.4. Effect of Different Surfactants on Enzymatic Hydrolysis*

To have applications in detergent industries, cellulase must be stable to various detergent ingredients, such as surfactants. Surfactants alter cell permeability of microorganisms which lead to increased protein secretion or surface effects on cell-bound enzymes. The detergents had various effects on different enzymes and most commonly used detergents were Tween-20, Tween-80, and SDS. Effect of these surfactants at three different temperatures (40°C, 45°C, and 50°C) at pH 4.8 for 120 h on treated groundnut fodder is shown in Figures 7-9. In this study, the hydrolysis was performed with the crude cellulases of *A. unguis* and using 1% alkali pretreated groundnut fodder as a substrate at pH 4.8 for 120 h. It was observed that addition of Tween-80 to the hydrolysis reaction had the highest positive influence on production of reducing sugars compared to other surfactants at all temperatures. However, addition of ionic surfactant (SDS) showed the negative effect on sugar yield at 40°C and 45°C temperatures. Hydrolysis of pretreated groundnut fodder with 1% (v/v) Tween-80 yielded maximum reducing sugar concentration, i.e., 7.16 g/L at 45°C and 50°C (Figures 8 and 9), whereas without surfactant (control) yielded reducing sugars of 2.24 g/L on 120 h of incubation at 50°C. Castanon and Wilke [38] similarly have found that a non-ionic surfactant Tween-80 increased the rate and extent of cellulose

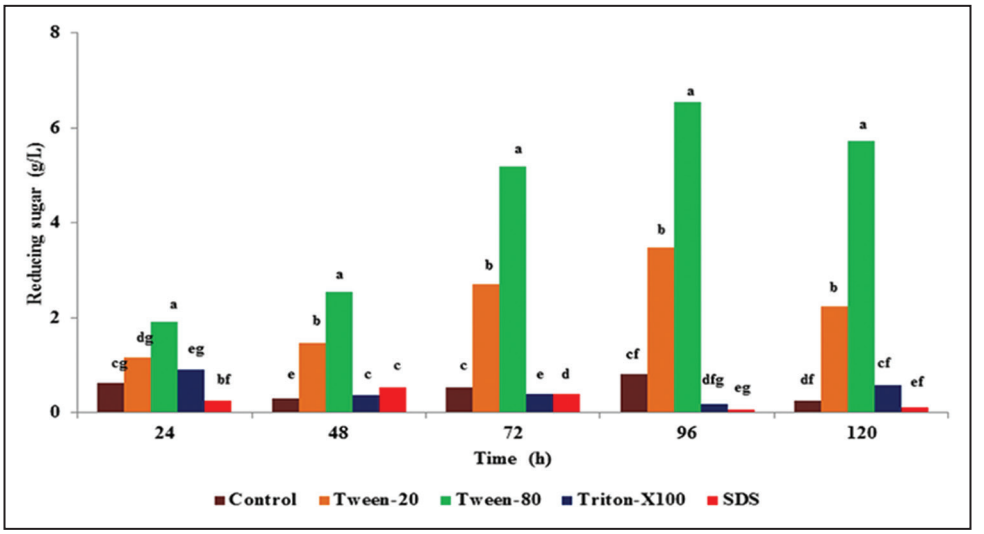


**Figure 7:** Effect of non ionic and ionic surfactants at 40°C on enzymatic hydrolysis of groundnut fodder.

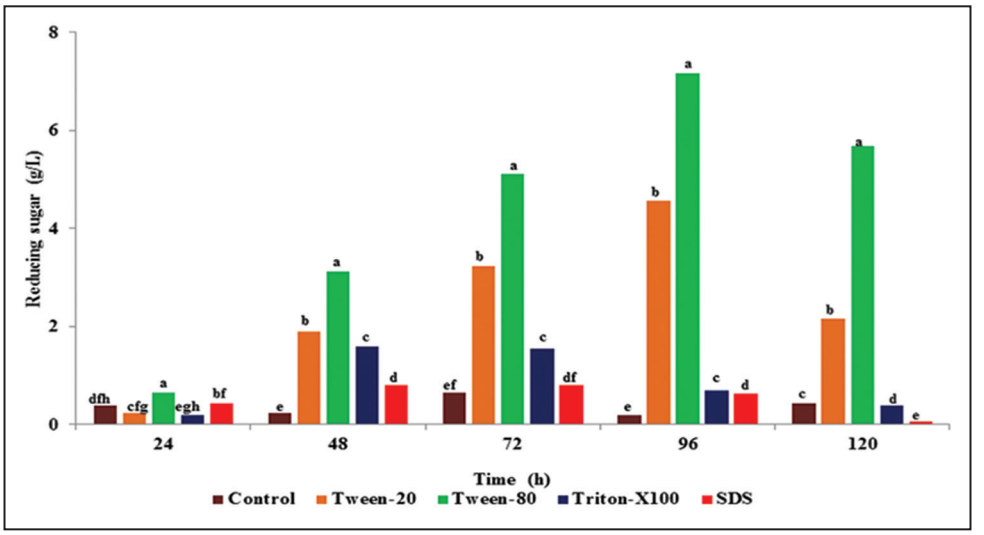


**Figure 8:** Effect of non ionic and ionic surfactants at 45°C on enzymatic hydrolysis of groundnut fodder.

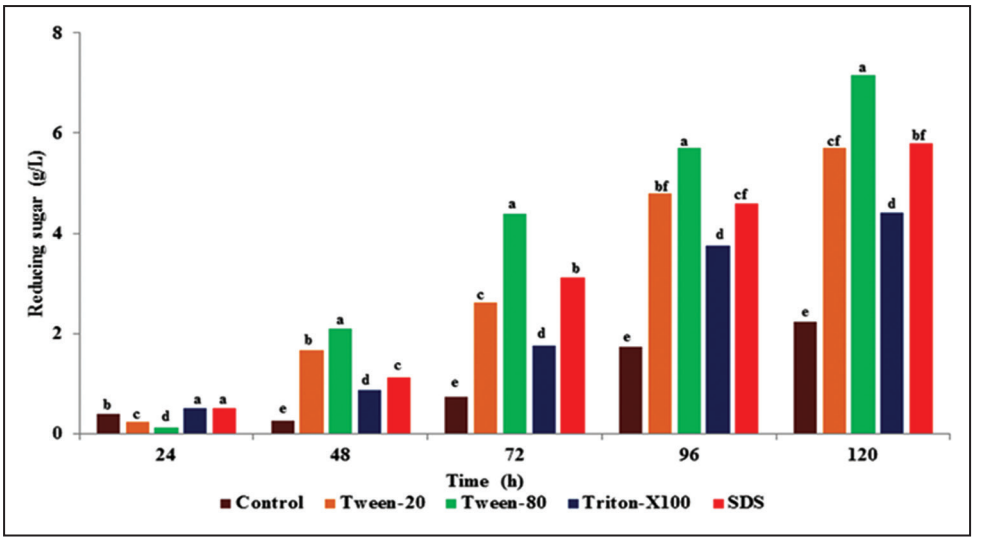


**Figure 9:** Effect of non ionic and ionic surfactants at 50°C on enzymatic hydrolysis of groundnut fodder.

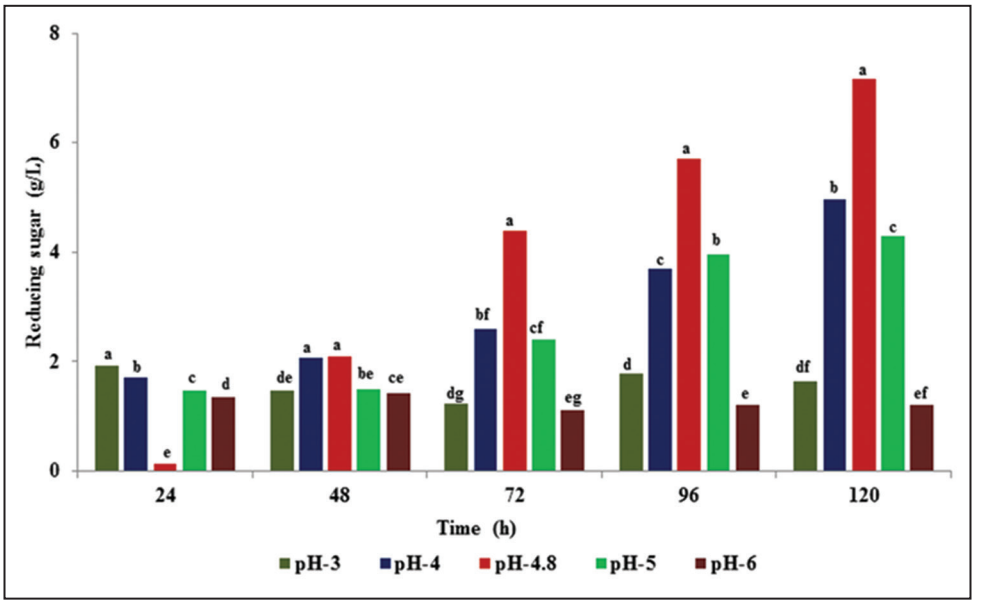


**Figure 10:** Effect of different pH and tween-80 on enzymatic hydrolysis of groundnut fodder at 50°C.

hydrolysis. This yield is lower than that reported by Sukumaran *et al.* [14] (26.3 g/L) who hydrolyzed rice straw pretreated with 0.1 N NaOH at 121°C with cellulase derived from two types of filamentous fungi grown in SSF. The yield was higher than that reported by Zhang and Cai [39] (2.23 g/L) for alkali pretreated rice straw with *Trichoderma reesei* ZM4-F3 cellulase produced by submerged fermentation. Thus non-ionic surfactants are more suitable for enzymatic hydrolysis of lignocellulosic than anionic surfactants. This might be due to the fact that anionic surfactants had a higher toxicity to enzymatic hydrolysis than the non-ionic surfactants. Denaturation of enzyme was the probable cause for the decreased conversion when anionic surfactants were used.

### *3.5. Influence of pH with Tween-80 on Enzymatic Hydrolysis*

pH with Tween-80 showed the positive effect on reducing sugar yields (Figure 10). Only pH (without addition of Tween-80) influenced the higher yield of reducing sugar released was 4.37 g/l at pH 4.8 (Figure 6). The higher yield of reducing sugar with 7.16 g/L was obtained at 120 h of incubation at pH 4.8 with 50°C (Figure 10). This indicates that pH with addition of Tween-80 shows the increase in sugar yield approximately 2-fold.

## 4. CONCLUSIONS

The current study clearly indicates that cultivation of *A. unguis* on 1% NaOH-treated groundnut fodder enhances the production of cellulases. Supplementation of non-ionic surfactants such as Tween-80 at 1% (v/v) showed the positive effect and ionic surfactant like SDS showed negative effect on reducing sugar yield. Temperature 50°C, pH 4.8, and 1 ml crude enzyme are the optimal conditions for maximum production of reducing sugars and pH with addition of Tween-80 improved the reducing sugar yield approximately 2-fold. The sugars released can be further utilized for the production of ethanol.

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