Enzymatic hydrolysis of complex agrowastes by *Bacillus cereus* ARA-12: A sustainable approach for biofuel production

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Abstract

Currently petroleum-based fossil fuels are the main source of the production of energy. The major issue is that all these resources are non-renewable. As the demand of fuel increases day by day, these non-renewable resources may deplete shortly and will not meet the supply criteria according to the requirements. To overcome this problem, the production of bioethanol using wastes biomass such as fruit peels, agricultural waste, municipal and kitchen waste etc. has gained considerable attention. As these agro-wastes mainly contain lignocellulosic biomass, lignocellulolytic bacterial cultures harness the full potential of these substances. In the present study, endoglucanase producing *Bacillus cereus* ARA-12 was isolated from soil samples. Fermentation parameters to produce endoglucanase were optimized. The optimum production of endoglucanase was achieved in the medium containing carboxymethyl cellulose (15g/L), yeast extract (30g/L), KH_2PO_4 (1g/L), K_2HPO_4 (1.45g/L), $MgSO_4$ (0.4g/L), $CaCl_2$ (0.05g/L) and $FeSO_4$ (0.00125g/L). The optimum temperature and pH of the medium were found to be 50°C and 8 respectively. The maximum enzyme production was achieved at agitation speed of 120 rpm after 20 hours of fermentation by using 5% inoculum. Maximum of 35.9 % bioethanol was produced by the action of endoglucanase enzyme on sugarcane bagasse whereas sweet potato, rice bran, banana peel, corn cob, potato peel and corn husk were also found to be potential raw material to produce second-generation biofuel.

Keywords: Agriculture wastes, *Bacillus*, Bioethanol, Endoglucanase, Second generation biofuel.

Introduction

Energy consumption and oil recourses utilization has been increased worldwide due to increase in population as well as industrialization [1]. According to the report of global economy US energy administration, oil consumption in Pakistan during the period of 1980 to 2014 has been raised from 104 thousand barrels to 450 thousand barrels per day, whereas production is just around 79.09 thousand barrels per day according to the data of 2020 (www.theglobaleconomy.com). Use of fossil fuel results in carbon dioxide (CO_2) emission which is a major contributor of climate change and global warming. In this context reduction in CO_2 emission is one of the key Sustainable Development Goals (SDGs) of United Nations (UN).

Disposal of agriculture waste in landfills increases land pollution and environmental

hazards as it harbors various pathogens [2]. Nevertheless, agricultural waste has a great potential to be converted into commercially valuable products [3]. Bioethanol, one of the most value-added renewable energy sources is manufactured by the microbial fermentation of sugary substances.

Therefore, the usage of sustainable and cheap starch and lignocellulosic substrates like bagasse, sawdust, and corncob has gained tremendous attention for renewable energy production [4]. Fruits and vegetable peels are also important substrates to produce bioethanol through fermentation [5].

High fiber content present in the starchy and lignocellulosic waste requires high temperature treatment along with various chemicals to convert them into fermentable form. This process results in the generation of various toxic compounds. Several studies have been carried out to produce bioethanol using microbial enzymes to avoid generation of toxic byproducts [6,7]. The process involves four main steps i.e. grinding of cellulosic biomass, enzymatic hydrolysis, fermentation process and distillation [8]. For the step of enzymatic hydrolysis, endoglucanase has been extensively studied because of their significance in the breakdown of cellulose containing materials in an eco-friendly manner to produce many valuable products like sugars, biofuels, chemicals, human nutrients, improved animal feed etc. [9,10].

In this study, *B. cereus* ARA-12 is studied accounting various parameters for the enhanced production of endoglucanase and subsequently various agriculture wastes are used to produce bioethanol.

Material and Methods

Isolation and identification of endoglucanase producer

Soil samples were collected from various coastal sites of Karachi and Baluchistan, Pakistan. 0.5gm of each soil sample was inoculated in 50 ml of nutrient broth containing 1% carboxymethyl cellulose. The inoculated broths were incubated at 37°C for 24 hours. After incubation, the samples were serially diluted from 10^{-1} to 10^{-4}. 0.5 ml from 10^{-4} dilutions of each sample was spread on the nutrient agar plates containing 1% carboxymethyl cellulose. The plates were incubated at 37°C for 24 hours. Isolated colonies were purified and checked to produce endoglucanase enzyme [11]. The colony ARA-12 that showed maximum zone of hydrolysis on CMC agar plate was selected for further study. The strain ARA-12 was biochemically characterized according to Bergey's manual of Bacteriology [12]. For species characterization, the genomic DNA was isolated and 16S rRNA analysis was performed [13]. The phylogeny of strain was determined using Mafft version 7.0 and ribotyped strains were submitted to GenBank.

Culture cultivation and enzyme production of endoglucanase

Six different previously reported media compositions were constituted to produce endoglucanase; medium 1 [11], medium 2 [14], medium 3 [15], medium 4 [16], medium 5 [17] and medium 6 [18]. Two sets of each medium were prepared and sterilized by autoclaving at 15 psi, 121°C for 15 minutes. Isolated bacterial strain was inoculated in each medium with a 5% (v/v) concentration. Set 1 was incubated at 37°C on shaking conditions at 120 rpm for 24 hours while set 2 was incubated at 37°C on static conditions for 24 hours. The cells were then centrifuged at 10,000 rpm for 10 minutes at 4°C. The cell free supernatant was used to detect the production of endoglucanase [19]. One unit of the enzyme activity was defined as the amount of endoglucanase that liberates one

micromole of reducing sugar per minute from the carboxymethyl cellulose under assay conditions [20]. The nutrient medium exhibiting maximum enzyme activity was taken up for further optimization.

Effect of temperature on the production of endoglucanase

Culture was grown at different temperatures ranging i.e.30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C and 65°C for 24 hours at 150 rpm. Cells were separated by centrifugation and the activity of endoglucanase was determined.

Effect of time on the production of endoglucanase

Maximum time interval to produce enzyme was investigated by cultivation of culture in the medium at 50°C for different time intervals at 18, 20, 22, 24, 26, 28, 30 hours. Endoglucanase activity in cell free broth was determined.

Effect of pH on the production of endoglucanase

To determine the optimum pH to produce endoglucanase, fermentation was carried out in the medium having pH 4, 5, 6, 7, 8, 9 and 10. Enzyme assay was performed.

Effect of carbon and nitrogen source on the enzyme production

To determine the effect of carbon containing compounds on the enzyme production 1% of various carbon sources i.e. glucose, maltose, sucrose, carboxymethylcellulose and lactose were used separately in the selected medium. For nitrogen source, 1% peptone, tryptone, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate yeast extract was used separately. After the selection of carbon and nitrogen sources, the concentrations of selected carbon as well as nitrogen source were also determined on 0-2% and 0-5% respectively. The enzyme assay was performed by standard assay method.

Effect of inoculums size to produce endoglucanase.

Inoculum size was determined by varying the concentration of inoculum from 1-7%. Each inoculated sample was incubated at 50°C for 20 hours. Enzyme activity was determined by the standard assay procedure.

Production of bioethanol

Preparation of feed stock

Production of endoglucanase was induced by the addition of 1% of different agriculture wastes including corn cob, corn husk, sweet potato peels, saw dust, wheat bran, sugarcane bagasse, potato peels, rice bran and banana peels in the optimized medium. Media was sterilized by autoclaving at 15psi, 121°C for 20 minutes. These flasks were marked as tests whereas medium in which carboxymethyl cellulose was added as carbon source was marked as control.

Production of bioethanol from agriculture waste

To produce bioethanol, combined enzymatic hydrolysis and yeast fermentation method was performed. At first, 10% (v/v) crude endoglucanase was added in media flasks of tests and control and incubated at standard assay conditions for 2 hours. Afterwards, 1 % of commercially available *S. cerevisiae* was added in it and incubated at 25°C for 24 hours. Each medium was then centrifuged at 10,1000 rpm for 10 minutes. The supernatant collected from each medium

was estimated for bioethanol production by potassium dichromate method. [21]

Results and Discussion

Selection and Identification of *Bacillus cereus* ARA-12

In the present study, 12 bacterial strains ARA-1 to ARA-12 showing zone of hydrolysis on nutrient agar plates supplemented with 1% carboxymethyl cellulose were isolated from soil. Among 12 isolated strains, the strain ARA-12 showed maximum cellulolytic index, therefore, selected for further studies (Figure 1 A, B).

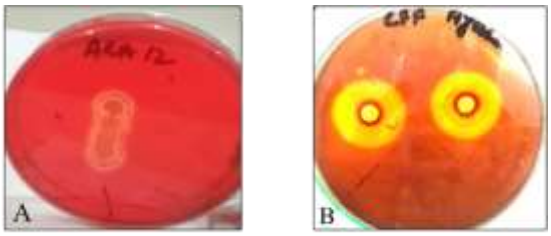

Figure 1: (A) Detection of endoglucanase enzyme produced by Bacillus cereus ARA-12. (B) Detection of endoglucanase enzyme produced by Bacillus cereus ARA-12 in the cell-free filtrate.

Microscopic analysis of the selected strain identified the ARA-12 as Bacillus sp. To confirm the identity, molecular characterization was performed by 16S rRNA sequencing. Results of sequencing revealed the strain as Bacillus cereus ARA-12. The sequence was submitted to GenBank with accession no. MK775015.1. The relationship of Bacillus cereus ARA-12 with other Bacillus species was determined by constructing the phylogenetic tree through Mafft version 7.0 (Figure 2).

Selection of cultivation medium

Bacterial growth as well as enzyme production is highly dependent on the composition of nutrients provided in the medium. In this study, six different previously defined media were used to produce endoglucanase. Results showed that among all, the highest endoglucanase production was achieved in medium 6 in both shaking as well as in static conditions. However, more enzyme activity was observed in the shaking conditions (Figure 3)

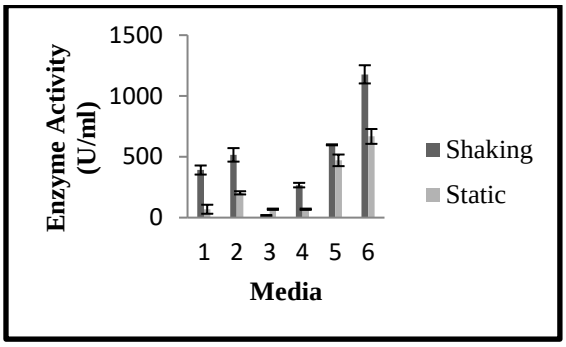

Figure 3: Effect of various reported media on the production of endoglucanase enzyme.

Among the different carbon sources tested, carboxymethyl cellulose was found to be the best for enzyme production. Yeast extract was added as a nitrogen source for the optimal growth of Bacillus cereus ARA-12. The major difference in this medium with other tested media was the composition of metal ions; sodium was not used in the production medium while all other five media contained sodium ions. It has been observed that sodium ions play a dual role in modulating the activity of endoglucanases. In some species, it inhibits the enzyme whereas somewhere it has been activating it [22]. The decline in the endoglucanase activity by the sodium ions has been reported by various researchers [23). Thus, it can be suggested that sodium ions inhibit the endoglucanase of B. cereus ARA-12 and maximum activity of enzyme in the medium 6 could be due to the absence of sodium ions.

Optimization of enzyme production parameters

To achieve the maximum production of endoglucanase from *Bacillus cereus* ARA-

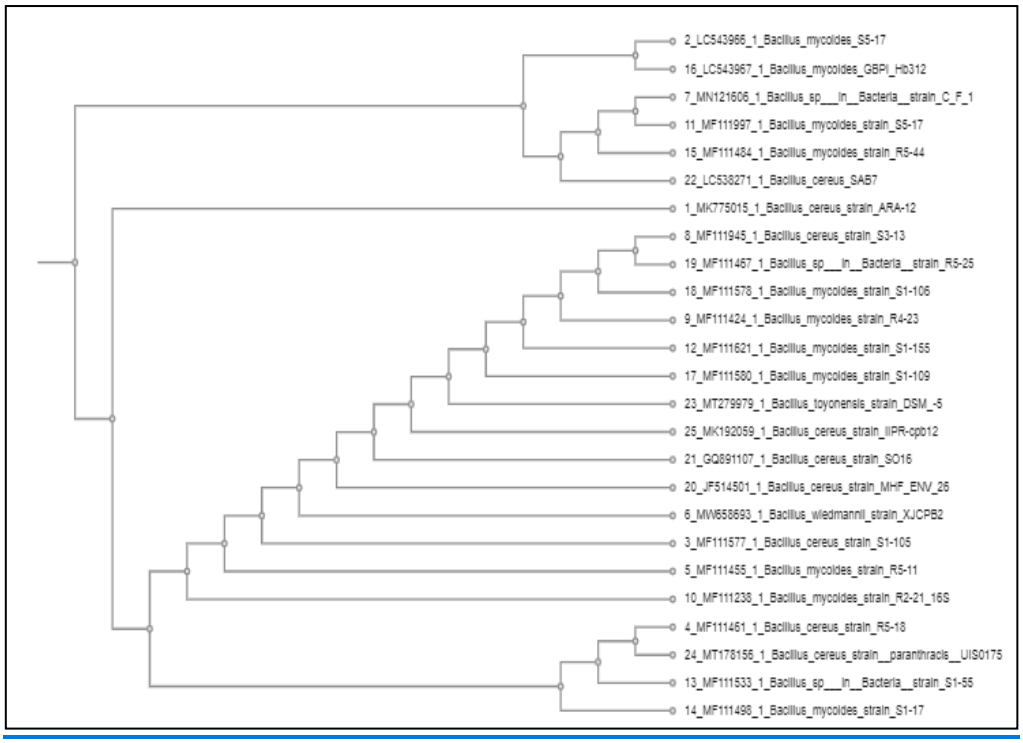

Figure 2: Phylogenetic tree of the 16S rRNA sequence generated by MAFFT version 7.0.

12, various fermentation parameters were optimized, and enzyme activity was determined after each parameter assessment.

Evaluation of optimum temperature for enzyme production

Production of endoglucanase is highly dependent on temperature of the fermentation media. Temperature affects the permeability of cell membranes by altering its structure and regulates the production of extracellular enzymes [11].

In the present study, it was observed that the optimum temperature for endoglucanase production was 50°C. Whereas, beyond 50°C, the stepwise decrease in the enzyme production was observed (Figure 4).

Similar results were also reported in which the endoglucanase activity started to decline after 50°C [24].

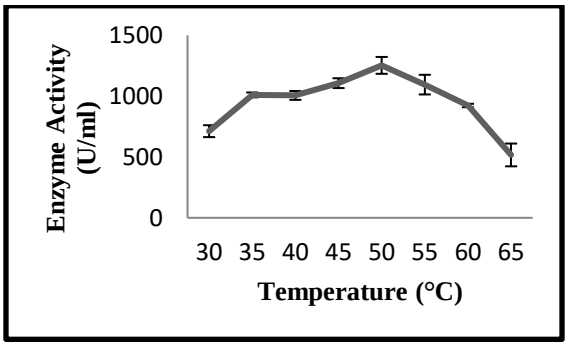

Figure 4: Effect of temperature on the production of endoglucanase enzyme.

Time course required for maximum endoglucanase production.

To optimize the time required for maximum endoglucanase production, the enzyme production was monitored at various time intervals. Results showed that at 20 hours of incubation maximum enzyme production

was obtained. However, there was no significant change in the enzyme production after the increase in incubation time (Figure 5). After 20 hours, the enzyme activity was decreased, and it may be due to the catabolic repression of the byproducts formed during the reaction. The decrease in enzyme activity after a certain time was also reported due to the consumption of nutrients by microorganisms leading to starvation conditions. [11]

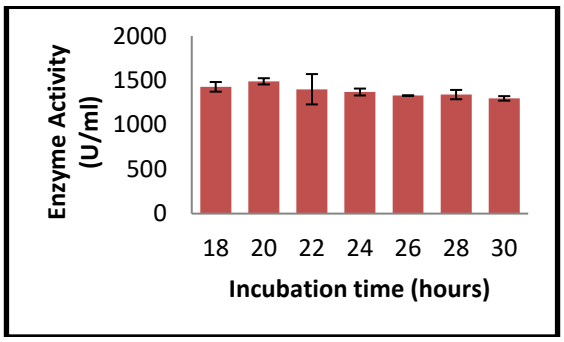

Figure 5: Evaluation of time course required to produce endoglucanase.

Evaluation of optimum pH for enzyme production

The pH of the medium is a physical factor that affects the transport of compounds across the cell membrane during fermentation which in turn maintains enzyme production and cell growth. It was observed that *Bacillus cereus* ARA-12 showed maximum endoglucanase production at pH 8; however, by increasing the pH towards more basic conditions, a decrease in the activity of endoglucanase was observed (Figure 6). pH also leads to the denaturation of the enzymes if they shift from their optimum value. Thus, the optimum pH stimulates microbial growth as well as the endoglucanase stability [25]. Various endoglucanases have been reported which are produced on alkaline pH of the production media [26].

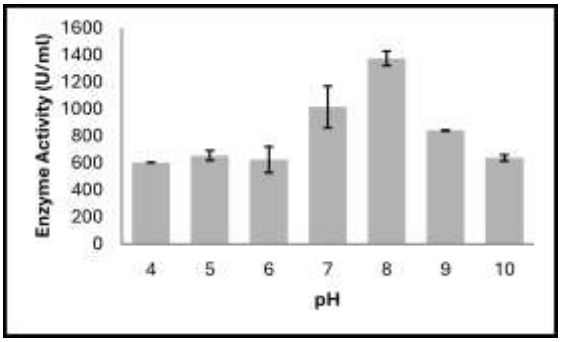

Figure 6: Effect of pH on the production of endoglucanase enzyme.

Evaluation of enzyme yield in the presence of various carbon and nitrogen sources

Carbon and nitrogen sources are the most valuable components of the fermentation media. These sources not only provide nutrition to the strain but also have a great influence on the production of metabolites from bacterial cells [27]. In this study, five different carbon sources were used in the production medium separately.

Results showed that the *Bacillus cereus* produced endoglucanase in the presence of production was obtained through all five carbon sources: however, maximum carboxymethyl cellulase at a concentration of 1.5% (Figure 7A and 7B).

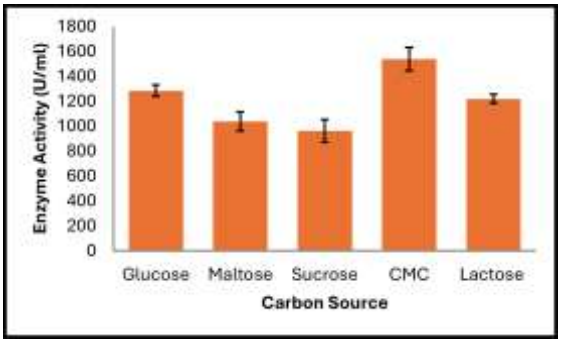

Figure 7(A): Effect of carbon source on the production of endoglucanase enzyme.

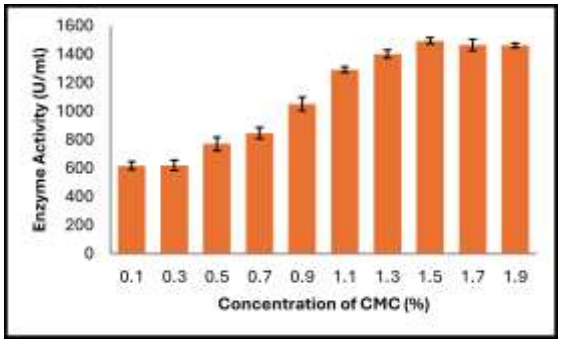

Figure 7(B): Effect of concentration of carboxymethyl cellulose on the production of endoglucanase enzyme

In the case of nitrogen source, seven various forms of nitrogen were used in the fermentation media. It was analyzed that *Bacillus cereus* ARA-12 produced endoglucanase by using all nitrogen sources whereas, the maximum endoglucanase production was observed in the presence of 3% yeast extract (Figure 8A and 8B).

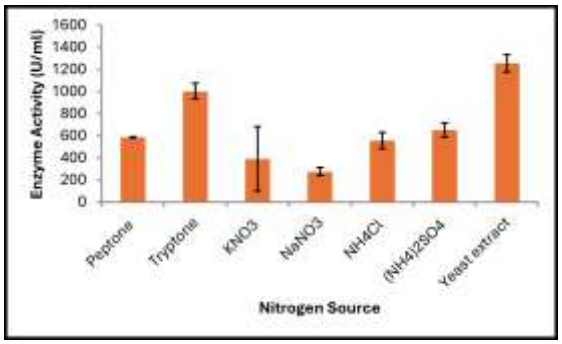

Figure 8(A): Effect of nitrogen source on the production of endoglucanase enzyme.

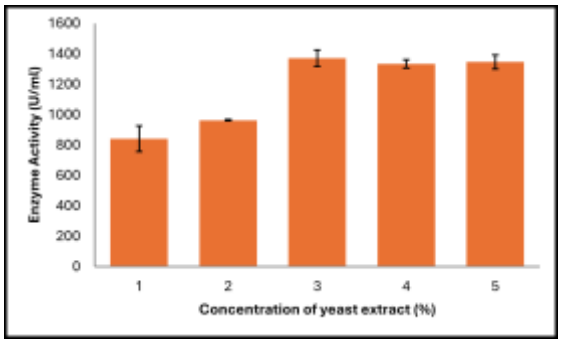

Figure 8(B): Effect of concentration of yeast extract on the production of endoglucanase enzyme.

Carbon and nitrogen sources for bacteria are crucial not only for growth but also for energy generation and biosynthesis. It has been reported that bacteria utilized various carbon and nitrogen sources, however, they selectively metabolized specific sources more as compared to other available sources [28].

Evaluation of inoculum size of *Bacillus cereus* ARA-12 on enzyme production

The adequate concentration of the bacteria inoculated in the media must be optimized to obtain maximum enzyme production. In the present study, the inoculum size was determined by varying the concentration of inoculums from 1-7%. Results showed that maximum enzyme production was achieved by using 5% (v/v) *Bacillus cereus* ARA-12 as inoculum. On the other hand, by increasing the concentration of inoculum above 5%, no further increase in the endoglucanase activity was observed (Figure 9). The initial lag phase of bacteria is reduced by providing the right number of viable bacteria per ml which also provides a favorable environment for bacteria for growth that ultimately increases the production of enzymes [29]. Basically, in case of batch culture, the numbers of cells are decreased with respect to time due to the depletion of nutrients during the fermentation process, thus, the size of inoculums plays major role in the product formation [30].

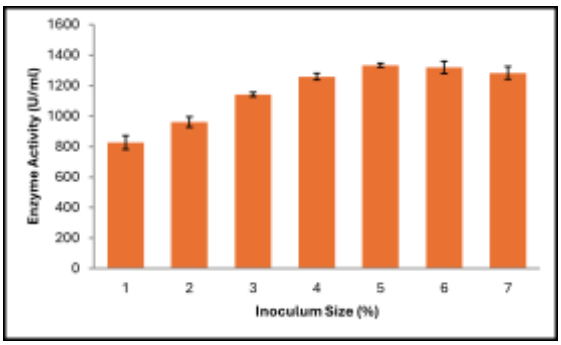

Figure 9: Effect of inoculum size on the production of endoglucanase enzyme.

Processing of agriculture waste by endoglucanase for bioethanol production

To produce bioethanol, pre-treatment of cellulosic biomass was performed by using crude endoglucanase enzyme. The sugars produced by the enzymatic treatment are fermented into ethanol by *Saccharomyces cerevisise* [31]. Results showed that 36 and 33% yield of bioethanol were obtained by using sugarcane bagasse and corn cob respectively, while rest of the agriculture wastes also showed the production of bioethanol (Figure 10). Basically, agriculture wastes are composed of lignocellulosic biomass and mainly consist of cellulose, hemicelluloses, and lignin; therefore, they are converted into monosaccharides and disaccharides like glucose and maltose by the action of endoglucanase enzymes [32].

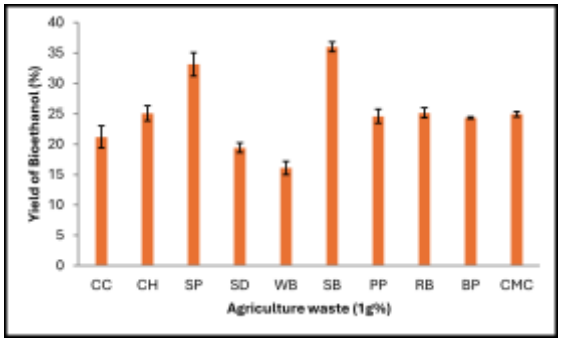

Figure 10: Utilization of agriculture wastes to produce bioethanol.

Various researchers also produced bioethanol by using agriculture wastes [33, 25]. It has been reported that 58.6% ethanol after the pre-treatment of waste with enzyme for 24 hours while in the present study 36% yield of ethanol was obtained in only 2 hours of enzymatic pretreatment of the feedstock [34].

CC: Corn cob, CH: Corn husk, SP: Sweet potato peels, WB: Wheat bran, SB: Sugarcane baggase, PP: Potato peels, RB: Rice bran, BP: Banana peels, CMC: Carboxymethy cellulose.

Conclusion

Bioethanol is the most popular alcoholic biofuel available in the current world market. Ethanol production by fermentation using renewable resource market. Ethanol production by fermentation using renewable resources represents an important alternative method for sustainable fuel production. Thus, in the present study endoglucanase producing *Bacillus cereus* ARA-12 was isolated and the production parameters for endoglucanase production were optimized. Efficient hydrolysis of various agriculture waste residues and the production of bioethanol were achieved by using endoglucanase produced by *Bacillus cereus* ARA-12. This could be beneficial to procide an eco-friendly method for the generation of biofuel at indigenous level Future studies will focus on the partial purification and immobilization of endoglucanase and the evaluation of immobilized enzyme to produce bioethanol.

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