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Research Article

Optimization of the Growth Condition and Molecular Identification of Two Bacterial Isolates from Egyptian Agricultural Wastes

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Abstract

Bacteria that produce cellulase were isolated from rice straw and sugarcane straw and screened using the Congo Red technique. 16S rDNA analysis was used to identify the highly cellulolytic isolates. CH1-Rice has homology with *Brevibacillus* sp., while CH5-Sugarcane has homology with *Klebsiella variicol*, according to phylogenetic tree analysis of their 16S rDNA gene sequencing information's. Bacterial optimum growth conditions were adjusted by manipulating nutritional and environmental parameters as temperature, pH, cellulose concentration, incubation time, salinity, and different carbon sources. The activity was estimated by measuring the turbidity of bacteria with a spectrophotometer. In conclusion, Two different bacterial strains were discovered anad isolated, and the optimal growth conditions for each were identified. These strains might be employed to transform plant waste into more beneficial substances.

Keywords: 16S rRNA; Cellulose Degrading Bacteria; Optimization; Saccharification; Bioethanol; Renewable Energy; Biodegradation

Introduction

Scientific advancements have provided humans with a variety of resources to use in order to improve the standard of living In order for a development to be termed sustainable, it "meets the demands of the present without harming future generations' ability to meet their own needs" [16].

The primary cause of the current global energy crisis and deterioration of the environment is the excessive consumption of fossil fuels, which are one of the nonrenewable resources and contribute significantly to greenhouse gas emissions [20]. Biofuels have been developed as alternative energy to help relieve this situation and are receiving a lot of attention because they are renewable.

Resource depletion is a result of the desire for non-sustainable alternatives and the expanding global population. In 2015, the global population was 7.3 billion people, with projections of 33% growth to 9.7 billion in 2050 and 53% growth to 11.2 billion in 2100 [7]. Meeting the energy demands of such a burgeoning population has been identified as one of humanity's greatest difficulties [22].

The most prevalent biomass and agricultural waste on the earth is called cellulose, and it is a polymer chain consisting of glucose units connected by β-1, 4 bonds [14]. The photosynthetic process produces cellulose waste, which is a large renewable bio-resource. It has a strong bioconversion potential to essential bio-products like ethanol. The ability to produce cheap ethanol will depend on the identification of new cellulase-producing bacteria [17].

The usage of cellulolytic and hemicellulolytic enzymes is one of the requirements for the biological production of industrial outputs from lignocellulosic wastes [13]. Cellulolytic bacteria (those that produce cellulase enzymes) can destroy cellulose. Several enzymes secreted by fungi and bacteria isolated from agricultural wastes decompose lignocellulosic biomass [3].

Cellulases are enzymes that convert short chains of cellobiose and glucose molecules into fermentable sugars, breaking down the insoluble cellulose polymer present in lignocellulosic feedstock [24].

In addition to increasing yield and economic viability, new cellulase-producing systems are being created in order to broaden the use of such systems by shifting toward more industrially applicable bacterial or fungal production systems. In order to improve the current method for creating cellulase enzyme, various enzymatic hydrolysis studies have concentrated on the production of enzymes with high enzymatic activity [11].

The objective of this study was to isolate bacterial strains that have the ability to degrade cellulose present in agricultural wastes such as Sugarcane and Rice straws, and optimize the condition for bacterial growth to have the best condition for large-scale production.

Methodology

Cellulose-degrading bacteria isolation and screening from rice and sugarcane sources

For cellulolytic bacteria isolation, the various agricultural sources were physically crushed and immersed in distilled sterilized water for 72 hours before being injected by 0.5 ml from the Supernatent on the isolation media called basal salt media with composition (KH_2PO_4 2g, $NaNO_3$ 2.5g, NaCl 0.2g, $MgSO_4$ 0.2g, $CaCl_2 6H_2O$ 0.1g in a litre). Colonies that appear on the isolation media were recultured on the identification media were incubated in static incubator (Red Line/RL-15-17848) for 7 days at 37°C. Utilizing cellulose agar media with a composition of (cellulose 2g, KH_2PO4 0.5g, $MgSO_4$ 0.25g, gelatin 2g, agar 15g, and dis water lL and at pH 6.8-7.2). Bacterial colonies capable of using cellulose as their sole source of carbon were selected for further analysis. Striking bacterial isolates on cellulose Congo-Red agar medium (KH_2PO_4 0.5g, gelatin 2g, $MgSO_4$ 0.25g, cellulose 2g, 0.2g Congo-Red, agar 15g, and dis water lL and at pH 6.8-7.2) proved their ability to breakdown cellulose [12].

Extraction of DNA, PCR amplification and 16S rRNA gene sequencing

The sarkosyl method was used to extract the DNA from the bacterial isolates [23]. A spectrophotometric analysis was used to quantify the DNA concentration. RedSafe DNA Stain-stained 1% agarose gels were also used to analyse DNA optically.

Applied Biosystems 2720 thermal cycler was used for PCR amplification of the 16S rRNA gene (ABI, Foster City, USA). Forward primer (27F: 5′- AGAGTTTGATCMT GGCTCAG-3′) and reverse primer (1492R: 5′- TACGGYTACCTTGTTACGACTT -3′) were used for 16S rRNA gene amplification [8].

50 ng of extracted DNA, 2µl of primer mix (10 µM of each primer), 1.5 µl of (25 mM) MgCl2, 5µl of (5X) PCR buffer, 0.5 µl of (10 mM) dNTPs, 0.5 µl of (50 unit/µl) By using nuclease-free water, the final volume was reduced to 25 µl and GoTaq® Flexi DNA Polymerase, Promega, USA, Cat. No. M8297.

Initial denaturation for 3 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 53°C, extension for 90 seconds at 72°C, and a final extension step for 7 minutes at 72°C. To resolve PCR results, 1% agarose gels dyed with RedSafe DNA Stain were utilized.

Purification of PCR products was performed using the QIA quick PCR purification kit (QIAgen Inc., USA) according to the manufacturer's instructions. The forward 16S rRNA primer was used to sequence purified PCR products at Macrogene, Inc., using an ABI 3730XL genetic analyzer (Applied Biosystems, Foster City, USA) (Seoul, South Korea).

The NCBI database was used to BLAST search partial 16S rDNA gene sequences [1]. The MEGA7.0 software was used to perform phylogenetic analysis on incomplete gene sequences [15].

Optimization of bacterial growth conditions

By culturing bacterial isolates at various physiological parameters such as temperature, pH, salinity, different concentrations of Cellulose, variable carbon source utilization, and incubation times, the optimal conditions for Cellulose usage by bacteria as a sole carbon source were investigated.

In this study bacterial isolates are selected that exhibit biodegradation ability to degrade cellulose, that exhibit greater biodegradability are employed to improve the conditions for cellulose's biodegradation rate.

Effect of different Temperature degrees

By transferring 20 ml of Cellulose liquid medium into 100 ml Erlenmeyer flasks containing Cellulose as the only source of carbon and energy then inoculating the flasks with two bacterial isolates, individually, and incubating them 20, 30, 40 and 50°C in a shaker incubator (biobase biodustry (china)/DC20171101-2) at 150 rpm. The growth was measured using a spectrophotometer and the turbidmetric technique at 650 nm.

Effect of different initial pH degrees

For biodegradation of Cellulose by bacterial isolates, several initial pH degrees are adjusted before autoclaving to (3, 5, 7, and 9) using a pH metre and solutions of HCL and NaOH with concentrations of 1N and 0.1N. Bacterial isolates were introduced to 20 ml cellulose liquid medium in a 100 ml Erlenmeyer flask, individually. The flasks were incubated at 30°C and 150 revolutions per minute. The turbid metric approach was used to determine the growth at various initial pH values using a spectrophotometer with a wave length of 650 nm.

Effect of different salt concentration (NaCl)

By employing Cellulose as a carbon and energy source, the effect of varied sodium chloride concentrations on the biodegradation rate of Cellulose was investigated. Cellulose medium with various salt concentrations (0.1%, 1%, 5%, and 10%) were prepared. Bacterial isolates corresponding to 0.01 OD_{620} were inoculated 20 ml cellulose liquid medium in 100 ml Erlenmeyer flasks. The flasks were incubated at 30°C and 150 rpm. The growth rate at various salt concentrations was measured using the turbid metric method with a spectrophotometer at 650 nm.

Effect of different concentration of Cellulose

Bacterial isolates were introduced to 20 ml cellulose liquid medium in a 100 ml Erlenmeyer flask at (pH 7) supplemented with different concentrations of Cellulose (0.1%, 0.2%, 0.3%, 0.4%). The flasks were incubated at 30°C and 150 rpm in a shaker incubator, and the growth at various concentrations of Cellulose was measured using a turbid metric method using a spectrophotometer at 650 nm.

Effect of different carbon sources

Carbon sources (1% w/v concentration) (glucose, fructose, sucrose, maltose) were introduced to the medium individually. Bacterial isolates were introduced to 20 ml cellulose liquid medium in a 100 ml Erlenmeyer flask. The flasks were incubated at 30°C. and 150 rpm. The growth of several carbon sources was measured using a spectrophotometer with a wave length of 650 nm.

Effect of different incubation periods degrees

Cellulose medium containing Cellulose was distributed among 100 ml Erlenmeyer flasks, each with 20 ml of media and two bacterial isolates inoculated. Shaker incubators were used to incubate the flasks at 30°C and 150 rpm. After 2, 4, 6, and 8 days of incubation, the growth was measured using the turbid metric method with a spectrophotometer at wave length 650 nm.

Results and Discussion

Cellulose-degrading bacteria isolation and screening from rice and sugarcane sources

Almost all of the microbial species in the environment have ability to grow on the basal salt media. Most of worldwide bioethanol is produced from sugarcane and remaining bioethanol produced from other crops such as sugarbeet, sorghum, wheat, rice [5]. By sampling from Agricultural waste samples from different soil source in Egypt, seven strains of bacterial species were isolated in regarding to their higher growth rate on culture media, all seven bacterial isolates sub cultured on Cellulose agar media that have only Cellulose as carbon source, only two strains of bacterial isolates showed best growth on Cellulose media, the two isolates sub-cultured on Cellulose agar media with Congo Red to confirm the ability of degradation by clear zone appearance.

Molecular identification by 16S rDNA sequencing

After isolation of genetic material and molecular identification of the two isolates. The NCBI database was used to BLAST search partial 16S rDNA gene sequences [1]. The MEGA7.0 software was used to perform phylogenetic analysis on incomplete gene sequences [15].

Sample name	Scientific name	Accession No.
CH1-Rice	*Brevibacillus sp.*	OP002026
CH5-Sugarcane	*Klebsiella variicola*	OP001638

Table 1: Illustrate the analysis data and identified bacterial sample with accession number according to data base.

Optimization of bacterial growth conditions

Effect of different temperature degree

Figure 1: Effect of different temperature degrees on the growth of two bacterial isolates grown in cellulose broth media as sole carbon and energy source.

Cellulose biodegradation occurs at a wide range of temperatures, and cellulose degradation normally slows as the temperature drops. The results revealed that two bacterial isolates, CH1-Rice and CH5-Sugarcane, degraded cellulose efficiently at a moderate temperature of 30°°C. similar with [6], Because metabolic activity, diffusion, and mass transfer were all promoted, biodegradation rates were much higher at moderate temperatures (15-30°C). Also These results are similar to those of [2] who found that the cellulase produced by *Pseudomonas fluorescence* was activated at 30 to 35ºC and the optimum temperature at 35ºC.

Effect of different pH degree

Figure 2: Effect of different pH values on the growth of two bacterial isolates grown in cellulose broth media as sole carbon and energy source.

The results showed that two bacterial strains, CH1-Rice and CH5-Sugarcane, accelerated breakdown at natural PH. Cellulose biodegradation is influenced by environmental factors such as pH. Most heterotrophic bacteria and fungi prefer a neutral pH [27]. Obtained data confirmed the findings reported by [19] who mentioned that pH 7 - 7.5 more suitable for optimization of cellulase production by *Bacillus subtilis* and *B. circulans.*

Effect of different salt concentration

Figure 3: Effect of change in NaCl concentration on the growth of two bacterial isolates grown in cellulose broth media as sole carbon and energy source.

The obtained results revealed that the breakdown of cellulose with two bacterial isolates is optimal when the medium is free of salt or slightly salty, and that the rate of Cellulose degradation falls as salinity increases. The decreased degradation rate is due to reduced metabolic activity; substrate availability to microorganisms in saline environments should be lower than in non-saline environments [10].

Effect of different concentration of cellulose

Figure 4: Effect of different concentration of cellulose on the growth of two bacterial isolates grown in cellulose broth media as sole carbon and energy source.

The concentration of Cellulose owing to induction is another factor that can alter the biodegradation rate. The degradation efficiency of Cellulose by two bacterial isolates, CH1-Rice and CH5-

Sugarcane, was investigated in this study at different concentrations. It was discovered that the degradation of Cellulose by two bacterial isolates rose gradually when the concentration of Cellulose was increased, obtained data confirmed the findings reported by [18] which state that cellulose was the best carbon source for utilization by organisms and by altering in the concentration the degredation rate increase until 1%.

Effect of different Carbon source on cellulase activity

Figure 5: Effect of different Sugar source on the growth of two bacterial isolates grown in cellulose broth media.

In this investigation, glucose proved to be the most effective carbon source for the synthesis of cellulase. Our findings diverged from those of [21] who found that lactose was the ideal carbon source for *B. subtilis,* a bacteria obtained from cow manure, to produce cellulase at its highest level. Maltose was discovered by [25] to be the most effective carbon source for *Bacillus sp*. However, according to [4]. CMC is essential for *Bacillus sp*. to produce cellulase. The best carbon source varies depending on the research project. This could be because of the studied strain, the carbon sources, or the isolation habitat. Utilization of carbon source beside the Cellulose is another crucifer properties for the degradation, it was found that the degradation was been higher when sugar was added in the medium compared to medium free from any another carbon source, and glucose considered as the best source.

The compound is converted by the microorganisms through metabolic or enzymatic reactions. It is based on two processes: growth and cometabolism, which is defined as the metabolism of a chemical when a growth substrate, which serves as the main source of carbon and energy, is present [9]. There is some evidence that the degradation of these high molecular weight compounds can be accelerated by the addition of another carbon source, such as sugars. It is generally accepted that high molecular weight compounds do not grow on them as sole carbon sources, so the organisms capable of degrading these compounds must obtain energy from other sources and cometabolize these recalcitrant polymers.

Effect of different Incubation period

Figure 6: Effect of different incubation period on the growth of two bacterial isolates grown in cellulose broth media.

The incubation periods were also investigated in this study by growing the two bacterial isolates CH1-Rice and CH5-Sugarcane in medium supplemented with Cellulose as a carbon and energy source, and the bacterial isolates were incubated for 8 days at a rotatory shaking condition of 150 rpm, with the optical density measured at different incubation period intervals (2, 4, 6, and 8) days. The results indicated that the two bacterial isolates were capable of demonstrating rapid growth during brief incubation periods and utilising the cellulose, and the data obtained supported the findings previously reported by [26] which state that the highest endoglucanase production in saccrification occurred after 192 hours.

Conclusion

Agricultural wastes contain a wide range of cellulolytic bacteria, the majority of which have not been isolated. We isolated two bacteria with remarkable degredation activity and identified them using 16S rDNA and biochemical assays. Isolates have been recognised as CH1-Rice *Brevibacillus sp.,* and CH5-Sugarcane *Klebsiella variicola.*

The isolated bacterial culture CH 5-Sugarcane shows result better than that isolated from Rice straw. Utilizing these ideal conditions, the two strains can be utilised in several industrial applications. Additional research on large-scale culture is required,

as well as improvements in other factors such inoculum size, inducer presence, and medium additives.

Conflict of Interest

There is no conflict of interest.

Bibliography

1. Altschul Stephen F., *et al*. "Basic local alignment search tool". *Journal of Molecular Biology* 215.3 (1990): 403-410.

2. Bakare M K., *et al*. "Purification and characterization of cellulase from the wild-type and two improved mutants of Pseudomonas fluorescens". *African Journal of Biotechnology* 4.9 (2005).

3. Bruce Thiago., *et al*. "Bacterial community diversity in the Brazilian Atlantic forest soils". *Microbial Ecology* 60.4 (2010): 840-849.

4. Deka Deepmoni., *et al*. "Enhancement of cellulase activity from a new strain of Bacillus subtilis by medium optimization and analysis with various cellulosic substrates". *Enzyme Research* 2011 (2011).

5. Dufey Annie. Biofuels production, trade and sustainable development: emerging issues. No. 2. Iied (2006).

6. Eriksson Mikael., *et al*. "Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic tundra soil". *Applied and Environmental Microbiology* 67.11 (2001): 5107-5112.

7. Faisal Muhammad and Aamer Saeed. "Sustainable approaches toward the production of bioethanol from biomass". *Sustainable Ethanol and Climate Change*. Springer, Cham, (2021): 15-38.

8. Frank Jeremy A., *et al*. "Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes". *Applied and Environmental Microbiology* 74.8 (2008): 2461-2470.

9. Fritsche Wolfgang and Martin Hofrichter. "Aerobic Degradation by Microorganisms: Biotechnology". *HJ Rehm, and G. Reed* (2008): 145-155.

10. Garg KL. "Effect of salinity on cellulolytic activity of some pneumatophore-inhabiting fungi of Sunderban mangrove swamps". (1982).

11. Garvey Megan., *et al*. "Cellulases for biomass degradation: comparing recombinant cellulase expression platforms". *Trends in Biotechnology* 31.10 (2013): 581-593.

12. Gupta Pratima., *et al*. "Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential". *International Journal of Microbiology* 2012 (2012).

13. Jourdiera Etienne., *et al*. "Simple kinetic model of cellulase production by Trichoderma reesei for productivity or yield maximization". *Chemical Engineering* 27 (2012).

14. Kamal Sajid., *et al*. "Insights on heterologous expression of fungal cellulases in Pichia pastoris". *Biochemistry and Molecular Biology* 3.1 (2018): 15-35.

15. Kumar Sudhir., *et al*. "MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets". *Molecular Biology and Evolution* 33.7 (2016): 1870-1874.

16. Kandakoglu Ahmet., *et al*. "Multicriteria decision making for sustainable development: A systematic review". *Journal of Multi-Criteria Decision Analysis* 26.5-6 (2019): 202-251.

17. Lee Sang-Mok and Yoon-Mo Koo. "Pilot-scale production of cellulase using Trichoderma Reesei rut C-30 fed-batch mode". *Journal of Microbiology and Biotechnology* 11.2 (2001): 229-233.

18. Pushalkar Smruti., *et al*. "Production of β-glucosidase by *Aspergillus terreus*". *Current Microbiology* 30.5 (1995): 255-258.

19. Ray Arun K., *et al*. "Optimization of fermentation conditions for cellulase production by Bacillus subtilis CY5 and Bacillus circulans TP3 isolated from fish gut". *Acta Ichthyologica et Piscatoria* 37.1 (2007): 47-53.

20. Rempel Alan., *et al*. "Bioethanol from Spirulina platensis biomass and the use of residuals to produce biomethane: an energy efficient approach". *Bioresource Technology* 288 (2019): 121588.

21. Saraswati B., *et al*. "Cellulase production by *Bacillus subtilis* isolated from cow dung". *Archives of Applied Science Research* 4.1 (2012): 269-279.

22. Smalley Richard Errett. "Top ten problems of humanity for next 50 years". Energy and NanoTechnology Conference. Houston, TX: Rice University, (2003).

23. Sambrook Joseph., *et al*. "Molecular cloning: a laboratory manual". No. Ed. 2. Cold spring harbor laboratory press, (1989).

24. Taherzadeh Mohammad J and Keikhosro Karimi. "Enzymatic-based hydrolysis processes for ethanol from lignocellulosic materials: A review". *BioResources* 2.4 (2007): 707-738.

25. Teodoro Carlos Eduardo de Souza and Meire Lelis Leal Martins. "Culture conditions for the production of thermostable amylase by *Bacillus* sp". *Brazilian Journal of Microbiology* 31 (2000): 298-302.

26. Teixeira da Silva Vanessa de Cássia., *et al*. "Effect of pH, temperature, and chemicals on the endoglucanases and β-glucosidases from the thermophilic fungus *Myceliophthora heterothallica* F. 2.1. 4. Obtained by solid-state and submerged cultivation". *Biochemistry Research International* 2016 (2016).

27. Venosa Albert D and Xueqing Zhu. "Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands". *Spill Science and Technology Bulletin* 8.2 (2003): 163-178.