

Bioethanol Production of Cellulase Producing Bacteria from Soils of Agrowaste Field

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Abstract

With decades of studies on cellulose bioconversion, cellulases have been playing an important role in producing fermentable sugars from lignocellulosic biomass. Copious microorganisms that are able to degrade cellulose have been isolated and identified. The present study has been undertaken to isolate and screen the cellulase producing bacteria from soils of agrowaste field. Cellulase production has been qualitatively analyzed in carboxy methylcellulose (CMC) agar medium after congo red staining and NaCl treatment by interpretation with zones around the potent colonies. Out of the seven isolates, only two showed cellulase production. The morphological and molecular characterization revealed its identity as *Escherichia coli* and *Staphylococcus aureus*. The potential of organisms for bioethanol production has been investigated using two substrates, namely, paper and leaves by subjecting with a pre-treatment process using acid hydrolysis to remove lignin which acts as physical barrier to cellulolytic enzymes. Ethanolic fermentation was done using *Saccharomyces cerevisiae* for 24-48 h and then the bioethanol produced was qualitatively proved by iodoform assay. These finding proves that ethanol can be made from the agricultural waste and the process is recommended as a means of generating wealth from waste.

Keywords: Cellulases; CMC agar; Bioethanol; Iodoform assay.

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1. Introduction

Agricultural business or industry has been accosted to considerably contribute to the country's economy. Hitting corresponding with the expansion of agricultural trade is that the generation of biomass waste. More than 104.55 million ton of biomass squander has been delivered yearly. The gigantic sum recommended lignocellulosic squander materials from rural buildups are biggest inexhaustible repository and conceivably reasonable feedstock to be changed over different worth added items [1,2]. The target of this investigation is to assess the creation of bioethanol by cellulase producing microbes from agrowaste. The extent of this strategy in the microbiological industry of ethanol production is likewise a focal point.

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Advancements for transformation of lignocellulosic materials into fermentable sugars are being widely investigated. The natural interaction of separating cellulose into glucose includes the synergistic activity at any rate three unique compounds: (i) endo- β -1,4-glucanase (EC 3.2.1.4), (ii) exo-cellobiohydrolase (EC 3.2.1.91), and (iii) β -glucosidase (EC 3.2.1.21). These compounds are comprehensively called as cellulases. Endoglucanase and exocellobiohydrolase act synergistically to separate cellulose into cellobiose which therefore will be cut by β -glucosidase to glucose [3, 4]. Microorganisms like bacteria and fungi, that can secrete all or individual types of cellulases, corrupt cellulose have become the current interest of scientists [5]. Such cellulase degrading microorganisms disengaged from assortment of sources such as composting heaps, decaying agricultural wastes, soil, defecation of ruminants and manures [6]. Higher growth rate of bacteria and capability of these types of microbes to endure harsh environments have become a critical factor for the move of ebb and flow research pattern to spotlight on bacterial cellulase production [7].

Bioethanol is an inexhaustible fuel and it is essential to tackle the issue of bioethanol creation to halfway supplant fossil-inferred energizes or fuels. The worldwide creation of bioethanol rose from 50 million m³ in 2007 to 100 million m³ in 2012 [8,9]. Bioethanol production from agro waste is a promising and upcoming innovative strategy. The whole process has a few constraints that include the transport and pretreatment techniques of biomass. Suitable fermentation techniques after enzymatic saccharification can improve the effectiveness of the entire interaction [10].

In this study, the prospective of potent bacterial strains for bioethanol production was investigated using two substrates, namely, paper and leaves by subjecting with a pretreatment process using acid hydrolysis to remove lignin which acts as physical barrier to cellulolytic enzymes. Ethanolic fermentation was done using *Saccharomyces cerevisiae* culture for 24-48 h and then the bioethanol was produced and estimated. Thus, the green strategy of biofuel production using renewable raw materials likewise in the present study paves the way to a cost effective and eco-friendly approach for overcoming the pollution in the current scenario.

2. Materials and Methods

2.1. Sample collection and isolation of bacteria

Soil sample was collected from agrowaste field at Kochi, Kerala. The isolation of cellulase producing bacteria was done by standard serial dilution method [11]. One gram of the soil sample was measured and mixed with 9 mL of sterile distilled water. The soil suspension was shaken vigorously under room temperature and the serial dilution was carried out up to the 10⁻⁶ dilution. Aliquots (0.1 mL) of 10⁻², 10⁻⁴ and 10⁻⁶ were spread plated on nutrient agar media. Inoculated plates were incubated at 37°C. After the incubation, morphologically distinct bacteria were selected for further analysis.

2.2. Screening of cellulase producing organisms

The colony isolated from nutrient agar plates were sub-cultured in carboxy methyl cellulose (CMC) agar media by spread plate technique [6]. The plates were incubated at 37°C for 24 h. To visualize the hydrolysis zone, the plate was flooded with an aqueous solution of 0.1% congo red for 15 min and washed with 1 mL sodium chloride (NaCl) [12].

2.3. Identification of potent cellulase producers

2.3.1. Colony characterization

The colonies characteristics such as shape, size, margin and pigmentation were noted for the colonies obtained on nutrient agar plates.

2.3.2. Gram's staining

A thin smear of the bacteria was made on a clean glass slide and were heat fixed. The smear was flooded with crystal violet and it was washed off after 1 min with distilled water. The smear was then flooded with Gram's iodine (for 1 min) that act as mordant and washed it off with alcohol. Then the flooded smear was safranin for 1 min and washed it off with distilled water. It was air dried and observed under microscope with 100X magnification using oil immersion. Gram-positive bacteria appear as purple color and Gram-negative bacteria will be seen in pink color.

2.3.3. Motility

A loopful of culture broth was placed on the center of the coverslip with paraffin on four corners. Immediately the glass slide is lifted and turned around. The drop of bacterial suspension "hangs" on the lower surface of the cover slip. The drop is then observed under the low power (10x) dry objective of the compound microscope. The edge of the drop must be focused. The bacteria tend to accumulate on the edge of the drop. Once the edge is located, it is then observed under the 40x high power objective [13].

Genomic DNA of potent isolate was isolated and purified [14]. 2 mL of microbial culture was spun at 12000 rpm for 10 min (4 °C). The supernatant was discarded and the same step was repeated. The supernatant was discarded finally and 875 µL of Tris EDTA buffer was added and vortexed. Then it was re-suspended. 5 µL of proteinase K and 100 µL of Sodium Dodecyl Sulphate (10 %) were added and mixed gently. 1 mL of Phenol: Chloroform mixture (1:1) was added and mixed gently. The mixture was spun at 12000 rpm for 10 min (4 °C). The supernatant was then transferred to a fresh tube and pipetted it out till it reached the interface. The above 2 steps were repeated with chloroform twice. The supernatant was transferred and 0.1 volume sodium acetate (5 molar, pH – 5.2) was added. Double volume of isopropanol was added and then stored at -20 °C. The tubes were kept at -20 °C overnight for 1-2 h. Final centrifugation was done

at 8000 rpm for 10 min. The tubes were washed with 500 μ L of ethanol and then spinned at 8000 rpm for 2 min. The ethanol was poured out and air dried. DNA was dissolved in TE buffer (50 μ L). Final tubes with DNA were stored at 4 $^{\circ}$ C.

2.3.4. Agarose gel electrophoresis for DNA quality and quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1 μ L of 6X gel-loading buffer (0.25 % bromophenol blue, 30 % sucrose in TE buffer pH-8.0) was added to 5 μ L of DNA. The samples were loaded to 0.8 % agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 μ g/mL ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.3.5. PCR analysis

PCR amplification reactions were carried out in a 20 μ L reaction volume which contained 1X PCR buffer (100 mM Tris HCl, pH-8.3; 500 mM KCl), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5 mM MgCl₂, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/mL BSA, 4% DMSO, 5 pM of forward and reverse primers and template DNA.

2.3.5.1. Primers used

Target	Primer pair	Direction	Sequence (5' \rightarrow 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The conditions used for PCR amplification are listed

Sl no	Step	Temperature	Time
1	Initial Denaturation	94 $^{\circ}$ C	1.5 min
2	Denaturation	94 $^{\circ}$ C	30 s
3	Annealing	56 $^{\circ}$ C	30 s
4	Extension	72 $^{\circ}$ C	2 min
5	Final Extension	72 $^{\circ}$ C	10 min

*Steps 2, 3 & 4 are repeated in 30 cycles

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

2.3.5.2. Sequence analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 [15].

The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm [16].

2.4. Cellulase assay using DNS method [17]

2.4.1. Preparation of standard.

1 mg of glucose was dissolved in 100 mL of distilled water.

2.4.2. Working standard preparation

Citrate buffer is made as follows: Citric acid is prepared by measuring 2.101 g of citric acid in 100 mL distilled water. Sodium citrate solution 0.1 M is prepared and dissolved 2.941 g of sodium citrate in 100 mL distilled water. 46.5 mL of citric acid, 3.5 mL of sodium citrate solution are taken and made up to 100 mL with distilled water. pH of the solution was adjusted to 2.5.

6 test tubes were taken and labelled as s1 to s5 and 1 test tube was labelled as blank. Appropriate volume of the working standard was pipetted out into different test tubes. The culture was centrifuged in 10000 rpm for 20 min and supernatants were collected. 1 mL of substrate was added in four test tubes followed by citrate buffer. Incubation was done in water bath at 50°C for 10 min. 1 mL of DNS reagent was added in all test tubes. Again, incubation was done in water bath at 90°C for 5 min. The absorbance was measured at 540 nm calorimetrically.

2.5. Production of bioethanol [18,19]

Sample preparation was done as initial step. Cellulosic material was used as feed stocks. Paper and leaves were grinded using motor and pestle with 0.9% NaCl. 2.0 g of feed stock of each substrate (leaves, grass, and paper) were measured and placed in three different glass bottles and labelled accordingly. A fourth glass bottle was labelled as control without any feedstock.

2.5.1. Pre-treatment

50 mL of hot distilled water was added in the glass bottles and swirled to mix. The caps were loosened from bottles and incubated in the hot water bath for 30 min. After incubation, the tubes were cooled down to room temperature.

2.5.2. *Enzymatic digestion*

2 mL of cellulase enzyme producing culture supernatant was added to all bottles and incubated at 37°C for 24 h. The tubes were allowed to cool to room temperature.

2.5.3. *Fermentation*

2 g of active yeast (*Saccharomyces cereveciae*) was added to each of the bottles and swirled to mix. An airlock was given to the top of the tubes. The air lock allows carbon dioxide to escape, keeping the pressure low in the bottles. It was again incubated for 24 h at 37°C.

2.5.4. *Iodoform test*

The iodoform test was done to confirm the presence of ethanol in the bioethanol produced by the bacterial isolate. 10 drops of distillate and 25 drops of iodine along with 10 drops of NaOH were added in the test tube. After few min, cloudy formation, yellow precipitate and antiseptic smell confirm the presence of bioethanol in the test tubes.

3. Results and Discussion

3.1. *Sample collection*

Soil sample was collected from agrowaste field at Kochi (Fig. 1). Bacteria were isolated from the collected soil sample through serial dilution technique (Fig. 2). Seven morphologically different types of colonies were formed and thus used for further studies. The isolates were streaked onto nutrient agar plates to study the colony characteristics (Fig. 3). These isolates were preserved on nutrient agar slants at 4°C for further analysis.



Fig. 1. Soil sample.

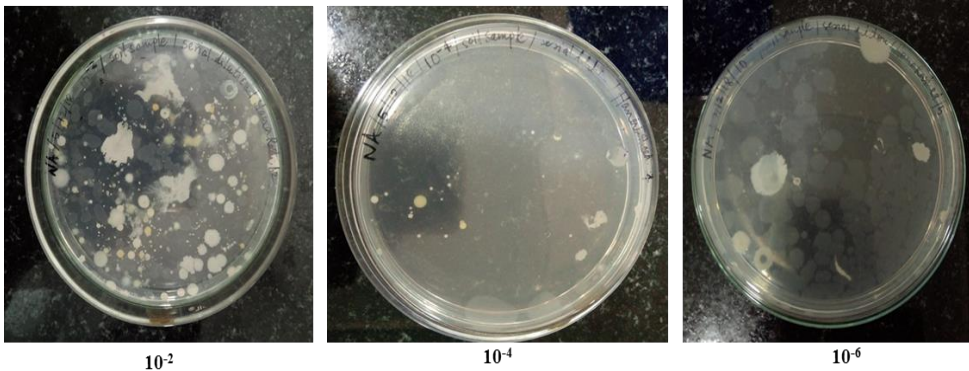


Fig. 2. Spread plate technique.

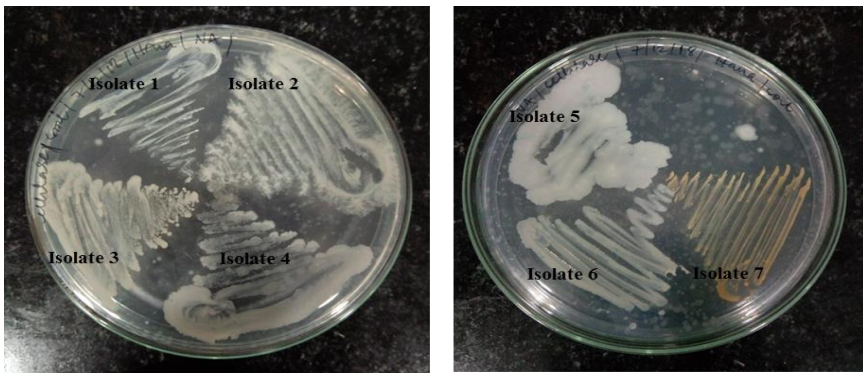


Fig. 3. Pure culture of seven bacterial isolates.

3.2. Screening of cellulase producing organisms

The isolated colonies were further spotted onto Carboxy Methyl Cellulose (CMC) agar medium for qualitatively analyzing the cellulase production. The colonies showing zone of degradation were observed. Two out of seven showed cellulase production and selected for further study (Fig. 4).

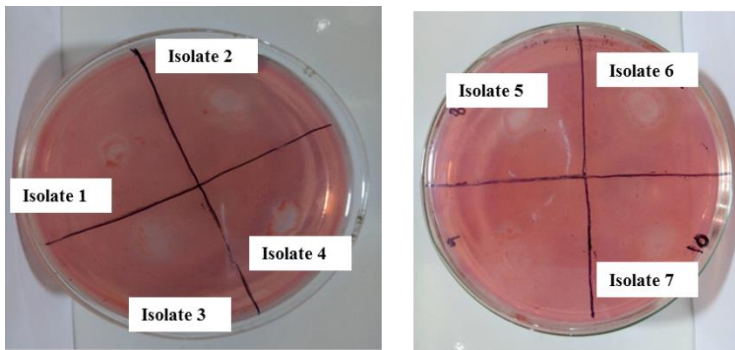


Fig. 4. CMC agar plate showing cellulase production by isolate 6 and isolate 7.

3.3. Identification of potent cellulase producers

Morphological colony characteristics of both the isolates are listed in Table 1. On Gram staining, the isolates 6 and 7 were observed as Gram negative rod and Gram positive cocci respectively (Fig. 5). The isolates 6 and 7 were observed as motile and non motile respectively (Fig. 6). Genomic DNA was isolated. A portion of the 16S rDNA was amplified using a primer pair for 16S rDNA (Fig. 7). The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm. The organisms 6 and 7 are thus identified as *Escherichia coli* and *Staphylococcus aureus* respectively in accordance with molecular characterization.

Table 1. Cultural characteristics of potent organism.

Isolate	Colony morphology on nutrient agar
Isolate 6	Large, opaque, raised irregular surfaced non pigmented colonies
Isolate 7	Small, opaque, round yellow pigmented colonies

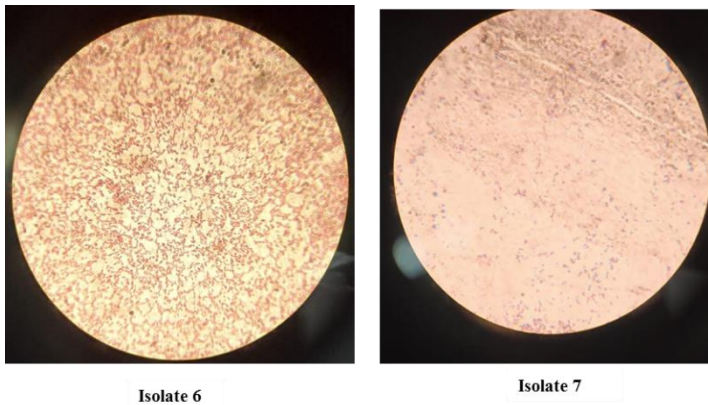


Fig. 5. Gram staining.

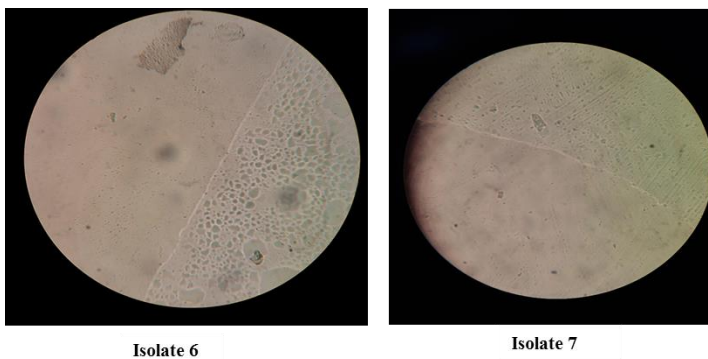


Fig. 6. Motility by wet mount.

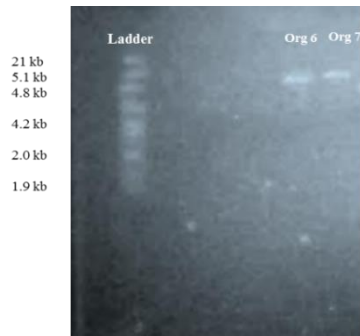


Fig. 7. Agarose gel showing the bands of Ladder- Lambda DNA / EcoR1/Hind III/ Double digest 1- Organism 6 and 7.

3.4. Production of bioethanol

For the production of bioethanol, cellulosic materials (paper and leaf) were used as feed stocks (Fig. 8). The iodoform test was done to confirm the presence of ethanol in the bioethanol produced by the bacterial isolate. From the assay, it was observed that maximum bioethanol production was observed by organism 6 followed by organism 7, when leaf was used as substrate. Comparatively least production of bioethanol was observed when paper is used as substrate for both the isolates (Fig. 9).

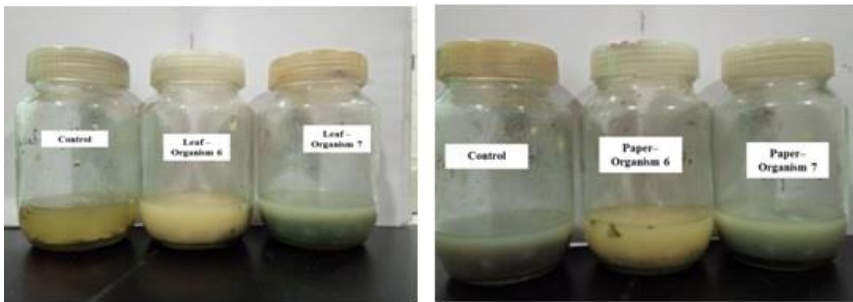


Fig. 8. Bioethanol production.

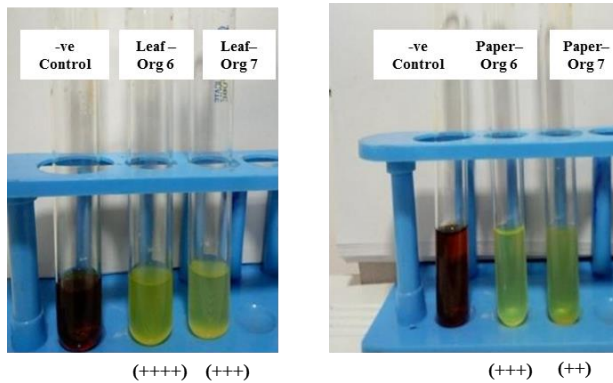


Fig. 9. Iodoform test.

Because of a high ethanol interest, the methodology for compelling ethanol creation is significant and has been grown quickly around the world. A few agrarian squanders are exceptionally bountiful in celluloses and the powerful cellulase chemicals do exist broadly among microorganisms. As needs be, the cellulose corruption utilizing microbial cellulase to create a minimal effort substrate for ethanol creation has pulled in more consideration. In an investigation, the cellulase delivering bacterial strain has been detached from rich straw and distinguished by 16S rDNA grouping examination as *Acinetobacter sp.* KKU44. This strain can develop and show the cellulase movement [20]. Fossil fuel sources like oil, coal, and gaseous petrol have added to the extraordinary expansion in the degree of ozone harming substances in the Earth's air [21]. This problem has resulted in the search for alternative energy sources that are environmentally friendly. In this study, two out of seven showed cellulase production and identified to be *Escherichia coli* and *Staphylococcus aureus*.

A work on bioethanol production from agrowaste studied the advantage of SSF over SMF, and successfully isolated cellulase producing organisms such as *Z mobilis* [6]. A work in line with is the treatment of municipal waste with different dilution of sulphuric acid (H_2SO_4). The hydrolysis of the sample measured in the form of reducing sugars showed that dilution had profound effect on hydrolysis. The hydrolysis or decomposition of the waste was acquired at 0.25 % of the corrosive weakening while most extreme was observed to be 0.75 %. Albeit the higher weakening was productively hydrolyzed the waste yet that diminished with higher grouping of the acids [22]. The investigation of Di Pardo 2000 [23] was on a similar subject. It was shown that microscopic organisms present an alluring potential for the misuse of cellulases and hemicellulases because of their quick development rate, protein intricacy and outrageous living space changeability. The improvement of quick and solid techniques for the screening of cellulases from microorganisms inside cold conditions will permit a more prominent number of novel bacterial cellulases to be disconnected with reason for modern use.

It was additionally tracked down that none of the chemicals separated to date, are completely impervious to the unforgiving ecological conditions utilized in the bioconversion cycle like high temperature, acidic or potentially salt pretreatments. In any case, these novel proteins can be additionally designed utilizing accessible information on compound construction and capacity through normal plan [24,25]. It was examined that cellulases can be utilized for natural waste bioconversion in biofuel industry, bio-compost industry and for production of synthetics. This should also be able to withstand mechanical conditions like temperature, pH and so forth and in this investigation, *Pseudomonas sp* was confined from urban waste after 72 h of maturation in CMC containing medium. For the production of bioethanol, cellulosic materials (paper and leaf) were used as feed stocks. The Iodoform test was done to confirm the presence of ethanol in the bioethanol produced by the bacterial isolates. From the assay, it was observed that maximum bioethanol production was observed by *E. coli* followed by *S. aureus*, when leaf was used as substrate. Comparatively least production of bioethanol was observed when paper is used as substrate for both the isolates.

There are reports on the production of cellulase by *Bacillus cereus* strains and their potential in the production of bioethanol [26,27]. To achieve maximum cellulase production, the cultural conditions of the incubation medium were optimized by studying the effects of pH, temperature, substrate loading, inoculum concentration and nitrogen on the production of cellulase. The results obtained showed marked effects of all these parameters on enzyme production. Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives pH of the medium and presence of inducers are important parameters for the optimized production of cellulase enzymes. Other researchers have also reported cellulase production enhancement by the optimization of cultural conditions [28-32]. Thus, in the present study, quantitative estimation and optimization of the production of bioethanol with appropriate substrate, i.e, leaf, are required for the pilot scale and large-scale business.

4. Conclusion

Because of expanding populace and industrialization, the interest of energy is expanding step by step. At the same time, the overall bio-ethanol creation is expanding continually. Creation of biofuels from sustainable feedstocks has caught extensive logical consideration since they could be utilized to supply energy and elective fills. Production of biofuels from renewable feedstocks has captured considerable scientific attention since they could be used to supply energy and alternative fuels. Seven morphologically different types of colonies were isolated through spread plate technique and were streaked onto nutrient agar plates for further study. The isolated colonies were further spotted onto Carboxy Methyl Cellulose (CMC) agar medium for qualitatively analyzing the cellulase production. Two out of seven showed cellulase production and selected for further study. After morphological and molecular identification of the isolated organisms 6 and 7 were identified as *Escherichia coli* and *Staphylococcus aureus* respectively. For the production of bioethanol, cellulosic materials (paper and leaf) were used as feed stocks. After iodoform test, maximum bioethanol production was observed by organism 6 (*Escherichia coli*) followed by organism 7 (*Staphylococcus aureus*), when leaf was used as substrate. Comparatively least production of bioethanol was observed when paper is used as substrate for both the isolates. Future prospective includes the pilot scale production of bioethanol from potent bacterial strains in the study after optimization and standardization of important parameters.

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