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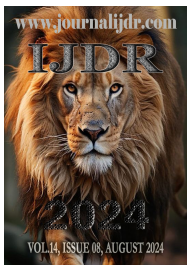

RESEARCH ARTICLE OPEN ACCESS

BIOETHANOL PRODUCTION FROM AGRO-INDUSTRIAL RESIDUES BY EXPLOITING CELLULOLYTIC BACTERIA: AN EVALUATION

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ABSTRACT

The development of biofuels from sustainable feedstocks has garnered significant scientific attention due to their potential to provide energy and alternative fuels. Cellulosic feedstock has gained attention as an attractive option for bioethanol production due to its greater ability to substitute fossil fuels and its relatively lower cost as a feedstock. With the help of a cellulolytic enzyme system, cellulose can be altered to glucose which is a multi-valued product, in a considerably economical and biologically favourable process. Many microorganisms that can degrade cellulose have been isolated and identified. The primary objective of this study was to identify and assess the cellulase-producing bacteria present in soil samples collected from sugar cane bagasse residue. The evaluation of cellulase production in carboxy methyl cellulose (CMC) agar medium was carried out qualitatively following Congo red staining and NaCl treatment. This was performed by analysing the zones surrounding the potent colonies. To evaluate the production of bioethanol at a laboratory level, the isolate showing the greatest enzyme activity was selected. Cellulase production has been facilitated by utilizing agro-industrial wastes such as rice bran, rice straw, sugarcane bagasse, wheat straw, and wheat bran, which have been used as substrates. A process of ethanolic fermentation was carried out by utilizing *Saccharomyces cerevisiae* for 24-48 hours. Subsequently, the bioethanol produced was subjected to quality evaluation by using titration, esterification, and iodoform tests. These results indicate the ability to produce ethanol from agricultural waste by cellulolytic bacteria, which makes it an attractive option for making money from waste.

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INTRODUCTION

Most agricultural residues of crop plant wastes, especially cereals, contain abundant lignocellulosic elements (Lynch, 1979). Cellulose is a primary component of lignocelluloses, consisting of long-chain polysaccharides composed of β (1,4) – linked glucose units. Lignocelluloses are formed by the interaction of cellulose with lignin, which is a complex polymeric molecule consisting of phenylpropanoid units. Utilising a cellulolytic system, cellulose can be enzymatically transformed into glucose, a versatile and valuable commodity, through a cost-effective and environmentally friendly method. Cellulolytic enzymes play an essential role in the natural process of biodegradation, where cellulolytic fungus, bacteria, actinomycetes, and protozoa efficiently break down plant lignocellulosic materials. These enzymes have been utilized in various industries to produce fermentable sugars, ethanol, organic acids, detergents, and other compounds. Cellulases offer a significant possibility to get substantial benefits from the utilisation of biomass (Wen *et al*., 2005). Cellulosic materials are sustainable organic resources that can be utilised for the synthesis of biofuels (Zhang and Lynd, 2004). The advancement of methods for the effective processing and utilisation of cellulose waste, such as inexpensive carbon sources, is thus of significant commercial importance. Cellulases, an enzyme system, breaks down cellulose. Cellulase is a complicated enzyme, that requires endoglucanase, exoglucanase, and β-glucosidase to work synergistically (Knowles *et al*., 1987; Zhang *et al*., 2006). This enzyme is generated by several microbes, mostly bacteria and fungi (Lederberg, 1992). However, fungi are well-known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al*., 2002). Cellulose-degrading bacterial species include *Clostridium*, *Trichonympha*, *Bacteroides*, *Actinomycetes*, *Ruminococcus albus*, *Succinogenes*, *Methanobrevibacter ruminantium* and *Butyrivibrio fibrisolvens* (Richmond, 1991; Selvankumar, 2011). The degradation of lignocellulosic material is a gradual process that can only be carried out by a limited spectrum of bacteria and fungi with specific taxonomic characteristics. The surge in oil costs, growing demand for

non-renewable resources in recent years, and environmental concerns due to climate change have sparked a rising fascination with biofuels. Biofuels are renewable, can substitute fossil fuels, reduce fossil greenhouse gas emissions and can be produced, where they are needed, to reduce the dependence on oil-producing countries. This study aimed to isolate cellulolytic bacteria from soil samples obtained from sugar cane bagasse waste and assess their cellulolytic activity. The production of bioethanol through simultaneous saccharification and fermentation of different substrates such as rice straw, rice bran, filter paper, sawdust, and sugarcane bagasse by co-culturing the isolated cellulolytic strains and yeast.

MATERIALS & METHODS

Sample Collection, Isolation: Soil samples of sugar cane bagasse residue were collected from Pammal, Tamil Nadu, Chennai, in sterile vials and kept at a temperature of 4ºC till they were used. Samples were serially diluted, with dilutions ranging from 10^{-1} to 10^{-6}. These dilutions were then inoculated onto Basal salt media by pour plate method and incubated for 48 hours at room temperature.

Screening of cellulolytic bacteria: The isolated colonies were grown on basal salt media supplemented with 1% carboxymethylcellulose (Hankin and Anagnostakis, 1977). The cultures were incubated at 30 ± 2°C until significant growth was recorded (Gautam *et al*., 2012) Then the Petri plates were flooded with Congo red solution (0.1%w/v) for 15 minutes. After pouring off the congo red solution, the plates were flooded with 1 M NaCl for 15 mins. After that zones of hydrolysis could be seen as clear areas, which indicate the cellulolytic activity of the organism (Shaikh *et al*., 2013). The clear zone around the colony was measured to select the highest cellulase producer (Gautam *et al*., 2012).

Maintenance of pure culture: The isolated cellulolytic bacteria were purified by the quadrant streaking method. The purified culture was maintained on 1.5% sterile agar (w/v) slants containing 1% CMC at 4˚C and subcultured fortnightly (Immanuel *et al*., 2006).

Growth study: The growth of cellulolytic bacterium was conducted by inoculating the isolated strain into flasks containing 250 ml basal salt broth containing 1% carboxymethyl cellulose. The culture was collected at every 10 hr of intervals for a period of 80 hr**.** The growth kinetics was determined by measuring the cell density at 660nm using a UV- Vis spectrophotometer.

Enzyme assay: Total cellulose activity was determined by measuring the amount of reducing sugar. To obtain crude enzyme, the culture was subject to centrifugation at 5000 rpm for 10 min. Cells were discarded and the supernatant was used as the crude enzyme. Cellulase assay was done by using a reaction mixture consisting of 0.5mL of crude enzyme, 1 mL of substrate solution (1% CMC in 50 phosphate buffer, pH 7.0), and making up the final volume to 3mL with distilled water. The reaction mixture was incubated at 30º C for 10 minutes. The reaction was stopped by heating the samples in a boiling water bath for 10 minutes. The reducing sugar produced in the reaction mixture was determined by dinitro- salicylic acid (DNS) method (Miller, 1959) with glucose as the standard. Enzyme activity was expressed in units (1 unit/ml = amount of enzyme which releases 1 µ mole glucose under the assay condition).

Effect of different concentrations of substrate (Carboxy Methyl Cellulose) on cellulase production: To select the optimum concentration of CMC, the isolate was inoculated in the production medium devoid of agar with 7 different concentrations of CMC (0.05%. 0.1%, 0.15%,0.2%, 0.25%, 0.3%, 0.35%) and without substrate.

Bioethanol production: The isolated bacterial strain was inoculated in basal salt broth for the production of cellulolytic enzyme and to initiate the saccharification process (Satheesh Kumar, 2009).
Agro-industrial residues such as rice bran, rice straw, sugarcane bagasse, wheat straw and wheat bran were used as different substrates for cellulase production. These substrates were used at three different concentrations (0.5%, 1.0%, and 1.5%). The culture was incubated at 37º C in a shaker at 100 rpm for 3 days. After completion of three days of incubation, the culture broth was conditioned for coculturing of *Saccharomyces cerevisiae* by the addition of filter sterilized salt solution (KH_2PO_4 - 0.4 g, $MgSO_4$ - 0.02 g, $CaCO_3$ - 0.05 g, and NaCl 0.01 g to 1 L culture broth). The simultaneous saccharification and fermentation were carried out at 27º C for 5 days in stationary conditions (Eklund and Zacchi, 1995; Lenziou *et al*., 1994). At the end of incubation, the culture broth was qualitatively tested for alcohol production using the K_2CrO_7 reagent test and also confirmed by reducing sugar assay, iodoform test, and esterification.

Determination of ethanol concentration in aqueous solutions: This method uses redox titration to find the concentration of ethanol in an aqueous solution. The ethanol is oxidised to ethanoic acid by reacting it with an excess of potassium dichromate in acid (College of Science, University of Canterbury).

Iodoform test : 10 drops of raw Bioethanol were taken in a test tube and 25 drops of iodine were added with 10 drops of NaOH. After a few minutes cloudy formations in the test tube confirm the presence of ethanol, it also gives a yellow precipitate and an antiseptic smell (Thomas *et al*., 2021).

Esterification test : A few ml of bioethanol and 1 ml of glacial acetic acid were added followed by 2-3 drops of concentrated sulphuric acid. Then the mixture was heated in a water bath for 10 minutes. The fruity smell indicated the presence of ethanol.

RESULTS AND DISCUSSION

Isolation and screening: Cellulose is the most abundant natural biomass on earth and the primary agricultural residue. It constitutes a significant portion of organic carbon present in soil and serves as the primary structural element in plants. Soil bacteria are essential for the recycling of organic carbon back into the ecosystem (Mswaka and Magan, 1998). The degradation of cellulosic material is an intricate process that requires the aid of microbial cellulolytic enzymes. Habitats with cellulosic substrates are the main resource for isolating bacteria capable of decomposing cellulose (Nutt *et al*., 1998). Several bacterial species have been identified with the ability to convert cellulose into simple sugars (Kasana *et al*., 2008), but there is still a need for newly isolated cellulolytic microbes. In this study, 5 bacteria were isolated from soil samples collected from sugar cane bagasse residue using serial dilution and pour plate method. Two of the five were eliminated because of similar colonial and morphological characteristics. Subsequently, the two resultant isolates were examined for cellulase activity by using tests on CMC agar. Their cellulolytic activities were confirmed by congo red method on CMC medium. Among these, the most potent one was chosen as an efficient cellulose producer and used for further study based on the diameter of the clear zone it produced on CMC agar plates (Plate 1).

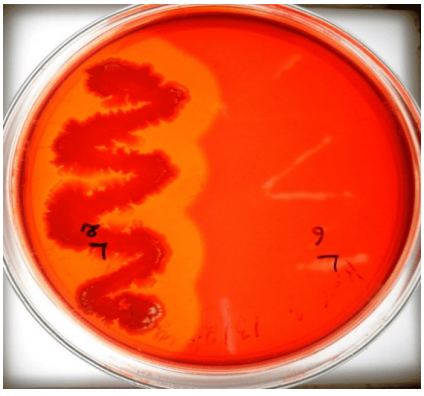

Plate 1. CMC agar plate showing cellulase activity

Purification of Bacterium: The most potent strain was identified and purified through primary screening. The purification process was carried out using the quadrant streaking technique. Distinct colonies were acquired and chosen for further studies.

Growth and enzyme assay: The growth kinetics of the isolated strain entered the lag phase immediately after inoculation. The stationary phase commenced at 40 hr and persisted until 70 hr, after which growth diminished. The production of cellulase reached the highest level at 2.7 U/mL after 70 hr of incubation (Fig 1).

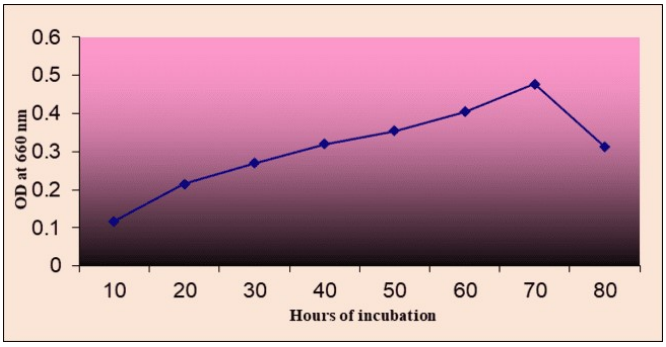

Fig. 1. Growth of the Isolated Bacterium

A cellulase assay was conducted to evaluate the efficacy of the cellulase enzyme. In this study, the isolated bacterium showed maximum cellulase production at 70 h of incubation. The cellulase production was seen in the declining phase of the growth. Selvankumar *et al.,* (Selvankumar, 2011) found that *Bacillus amyloliquefaciens* produced maximum cellulase within 72 h of incubation.

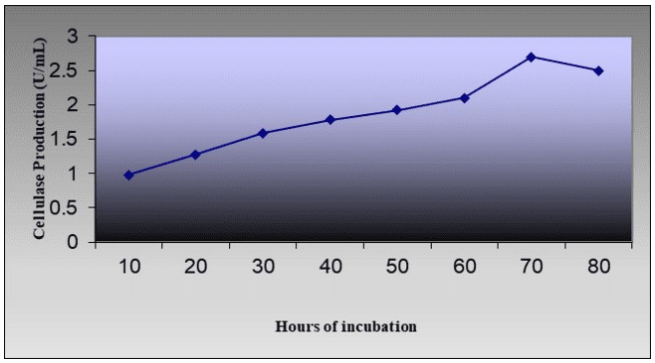

Fig. 2. Cellulase production at different time intervals

The optimum time for maximum enzyme production by various bacterial sp. has been reported to be quite variable. The extent of enzyme production in organisms is mostly influenced by the specific microbial strains and their genetic composition, as well as the cultural and environmental circumstances utilised throughout their growth (Bajaj *et al*., 2009). It has been stated that critical factors in maximizing cellulase production are the quality of the carbon source (carboxymethyl cellulose), pH, temperature, presence of inducers, medium additions, aeration, and growth period (Immanuel *et al*., 2006). Tiwari *et al*., 2017, (Tiwari *et al*., 2017) also found that *Streptococcus* and *Bacillus* sp. produced the highest level of cellulase from sugarcane bagasse within 72 h of incubation. This was due to its late stationary phase. Production of enzymes usually begins during the log phase of the growth and reaches its highest levels during the early stationary phase (Sudharhsan *et al*., 2007). The current strain exhibited the maximum growth at 70 hr of incubation after which the growth declined (Fig 1).

Effect of CMC as the substrate for cellulase production: The isolated bacterium showed less activity in the medium without the substrate, CMC. It showed a maximum activity of 2.8U/mL after 70 h incubation at 0.3% CMC (Fig 3).

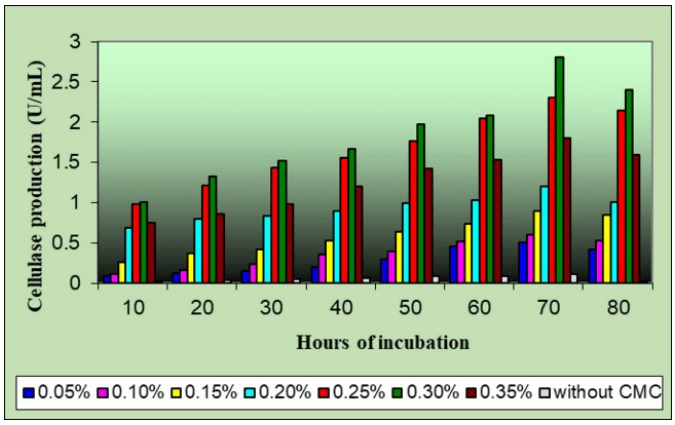

Fig. 3. Effect of different concentrations of CMC on cellulase production

The results are in agreement with those of Narasimha *et al.,* 2006 (Narasimha *et al*., 2006) who found that carboxymethyl cellulose was the best carbon source followed by cellulose for cellulose production. The increased synthesis of cellulase observed when CMC is used as a substrate is likely due to the activation of the enzymes. Paul and Varma, 1993 (Paul and Varma, 1993) reported the stimulation of endocellulase production using CMC stimulation.

Ethanol production using mixed cultures of bacteria and yeast

Determination of ethanol concentration in aqueous solutions: The experimental setup for simultaneous saccharification and fermentation of bacterial culture with *Saccharomyces cerevisiae* resulted in ethanol production (Plate 2). We found that the production medium with bagasse as a substrate produced up to 0.18% bioethanol. In sawdust, it produced up to 0.2%, and in filter paper, it produced up to 0.28% bioethanol (Fig. 4), which is more than the organism that grew in the medium with the other four substrates. This result demonstrates the high cellulolytic potential of the selected bacterial isolate for cellulose decomposition and fermentation for ethanol production.

Plate 2. Estimation of ethanol concentration in aqueous solutions by titration method

Iodoform test: The iodoform test is a method adopted for evaluating bioethanol production. The presence of ethanol was indicated by a yellow precipitate (Plate 3) (Prasad *et al*., 2023; Thomas *et al*., 2021).

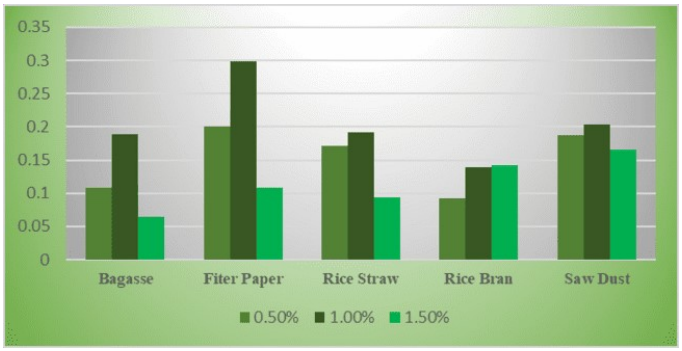

Fig. 4. Comparative study of bioethanol production in different substrates

Plate 3. Iodoform test

Esterification: To evaluate the bioethanol production in the culture broth, an esterification assay is conducted. The fruity smell indicates the presence of ethanol (Plate 4).

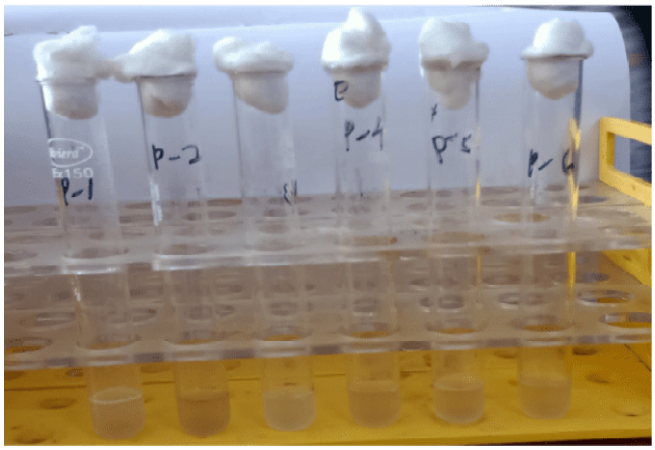

Plate 4. Esterification test

Several researchers have documented the cocultivation of bacterial strains with yeast sp and the simultaneous saccharification and fermentation of ethanol Ethanol is a byproduct of fermentation when simple sugars serve as the primary substrate. Yeast, a microorganism that converts glucose into ethanol through fermentation (Eklund and Zacchi, 1995; Lenziou *et al*., 1994). Ratledge, 1991 (Ratledge, 1991) states that *S. cerevisiae* is commonly used in hexose fermentation. It can produce ethanol from glucose and mannose in high sugar concentrations and anaerobic conditions, Various microorganisms such as bacteria, fungi and yeasts are utilized in ethanol fermentation processes. Yeasts are more favourable than other microorganisms for industrial fermentation because they have larger sizes, stronger cell walls, better development at low pH, less strict dietary needs, and greater resistance to contamination (Jeffries, 2006). There are many studies have been conducted on the process of alcohol production through yeast fermentation (Lark *et al.,* 1997).

CONCLUSION

"First generation" bioethanol refers to the process of converting sugar-based raw materials into ethanol, "second generation" refers to the use of lignocellulosic raw materials, and "third generation" refers to algal bioethanol. Bioethanol production from lignocellulosic biomass holds significant promise as an ecologically viable replacement for crude oil-based fuels. This approach can potentially address various agricultural byproducts that include cellulose, straw, wood trimmings, sawdust, bamboo, and other similar materials. In this study, we obtained the cellulolytic bacterium from soil samples of sugar cane bagasse waste using the serial dilution and spread plate method. The most potent strain is selected based on the hydrolytic zone produced on CMC agar plates. The bioethanol synthesis involved using various agro-industrial residues as substrates and subjecting them to quality evaluation through titration, esterification, and iodoform tests. Using filter paper as a substrate yields the highest measured bioethanol output of 0.2%. The future outlook entails the implementation of strain enhancement techniques to achieve high-volume bioethanol production, as well as the refinement and standardization of test conditions. However, ethanol produced from lignocellulosic biomass can address the current conflict between food and fuel production that emerged with the utilisation of first-generation biofuels. Moreover, it has the potential to make a substantial contribution to the generation of renewable energy.

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