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Bioethanol Production by Optimizing Cellulase Production from Bacteria

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Abstract : *Cellulose is a major constituent of plant cell wall and is the most abundant biological polymer on earth. The use of various cellulolytic microorganisms for the bioconversion of cellulose into valuable products has attracted worldwide attention. Our present work was aimed to isolate, purify and optimize the cellulase producing bacteria for bioethanol production. Optimization of the fermentation medium was carried out for maximum cellulase production and enzyme assay. The culture conditions like pH, temperature, incubation time, carbon sources, and nitrogen sources were optimized. The optimum conditions found for cellulase production and then for bioethanol production were 45°C at pH 4.5 with incubation time of 216 hours with peptone as a good nitrogen source and different substrates (coconut husk, rice bran, and pineapple peel). The yeast Saccharomyces cerevisiae was used for simultaneous saccharification and fermentation because it tolerates a wide range of pH with acidic optimum, and it is the preferred strain for industrial production of bioethanol.*

Keywords: *Isolation; Exoglucanase; Endoglucanase; Cellulase; Cellulose.*

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Introduction:

Cellulose is a linear polysaccharide of glucose residue with β-1, 4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products.

It is the most abundant biomass on the earth (Tomme et al. 1995). It is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere (Jarvis, 2003 and Zhang et al. 2004).

Cellulose, a polymer of glucose residues connected by beta 1, 4-linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature (Saha et al. 2006). Therefore, there is considerable economic interest to

develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources.

It is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1, 4-β-endoglucanase, 1, 4-β exoglucanase, and β-glucosidase or cellobiase. Endoglucanase is responsible for the random cleavage of the glycosidic bonds along a cellulose chain. Exoglucanase is necessary for the cleavage of the non-reducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and beta-1, 4-glucosidase hydrolyses cellobiose and water soluble cellodextrin to glucose (Shewale, 1982 and Woodward et al. 1983). Only the synergy of the three enzymes makes the complete cellulose hydrolysis to glucose (Ryu et al. 1980).

Extensive basic and applied research on cellulases revealed the commercial significance and industrial applicability of this enzyme. Cellulase has a wide range of application in a variety of sectors such as food, paper/pulp, pharmaceuticals, textiles, alcoholic beverages, malting and brewing, starch processing, biofuel production and leather etc. (Bhat, 2000).

Bioethanol is a form of alternative energy that can be produced from sugarcane, molasses and agricultural wastes. However, cost of raw material and cellulase in enzymatic hydrolysis are regarded as a major factor. Research and development to reduce the cost of bioethanol has been carried out from various aspects. Relatively large amount of bioethanol production tried to reduce cost at substrate level by using lignocellulosic materials as substrate, such as sugarcane, rice bran, coconut husk, as well as agricultural waste, wastepaper and leaf waste due to there being renewable, mainly unexploited, largely abundant, inexpensive resource having high content of readily convertible and fermentable sugar.

Keeping in view these facts about cellulase and its usability in industrial sectors, the present work aimed at isolation and screening of cellulolytic bacteria for the production of cellulase. Further the different parameters were also optimized keeping in view maximum bioethanol production from the selected isolated culture.

Materials and methods:

Soil sample from the surface was selected randomly from the garden area in sterilized polythene bags and then they were further processed within 24 hours of procurement.

Isolation: 0.1g of soil sample was serially diluted in 10 ml of sterilized normal saline upto 10^{-7}. Nutrient agar media (Peptone, 5 g; Beef extract, 3 g; Sodium chloride, 5 g; Agar 15 g; Distilled water, 1000 ml, pH 7.4±0.2) was prepared, autoclaved and poured into sterilized petri plate under sterile condition. Plates were left for solidification and were inoculated with the dilution of 10^{-5}, 10^{-6}, and 10^{-7} by spread plate method. Then the plates were incubated at 37°C for 24hours.

Different colonies obtained on NA plates, randomly six colonies (CB1, CB2, CB3, CB4, CB5, and CB6) were selected based on the cultural characteristics and were streaked on Nutrient agar slants and maintained for further use with periodic sub culturing.

Screening of cellulose degrading bacterial strains: The isolates that were streaked on NA slants were grown on carboxy methyl cellulose (CMC) agar media (CMC,10g; Dipotassium hydrogen phosphate,1g; Potassium dihydrogen phosphate,1g; Magnesium sulphate, 0.2g; Ammonium nitrate,1g; Disodium hydrogen phosphate,1g; Ferric chloride,0.05g; Calcium chloride,0.02g; Agar,20g; Dstilled water,1000ml, pH -7.0). The plates were incubated at 37°c for 48 hours. After incubation, the isolates were qualitatively and quantitatively tested for cellulase activity.

For qualitative test (clear zone) CMC agar media plate with isolated colonies were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1M NaCl (Apun et al., 2000). The strain showing a clear zone due to utilization of CMC was selected as a potential cellulolytic strain for further study.

Enzyme production and assay: For further quantitative estimation (amount of glucose released) of cellulase activity, each of the isolates was incubated in CMC broth for 24 to 48 hours separately. Then, centrifuged at 5000 rpm for 15 minutes. The supernatant was collected, which acts as crude enzyme, and stored at 4°C for further use. 0.1 ml of crude enzyme was taken in a test tube to which 1 ml of sodium citrate buffer was

added, further pinch of CMC was added in it. The test tube with sample was incubated at 50°C for one hour in incubator. At the end of incubation period 3 ml of DNS reagent (3,5-dinitrosalicylic acid,10.6g; Sodium hydroxide,19.8g; Rochelle salt (Sodium potassium tartrate), 306g; Sodium metabisulphite, 8.3g; Phenol, 7.6ml), added and then boiled in water bath for 5 minutes. After that test tube was cooled in ice cold water. Then O.D was taken at 540 nm.

Then a graph was plotted taking glucose as the standard to estimate the amount of glucose released or produced due to cellulase enzyme.

Maintenance of isolate: Most efficient cellulolytic strain was maintained by periodic sub culturing and kept at 4°C.

Characterization of isolate: The best bacterial isolate with maximum cellulase production was further identified on the basis of morphological characteristics.

Morphological characteristics : Culture was characterized morphologically by Gram's staining (Hans Gram) and other colony characteristics like colour, margin, texture, and elevation.

Optimization for maximum cellulase production:

pH : pH is an important factor affecting the microbial growth as well as enzyme production. Therefore, different pH values (4.5, 5.5, 6.5, 7, and 7.5) were taken to study their effect on cellulase production.

Temperature: Temperature is an important factor in a bioprocess for the production of extra-cellular enzyme. For the selection of optimum temperature for cellulase production, the isolated culture was incubated at different temperatures (26°C, 32°C, 37°C, 45°C, and 55°C) for 48 hours.

Incubation time: The effect of incubation time on cellulase activity was observed by incubating the isolate at 37°C for different time intervals and hours (24, 48, 72, 96, 120, 144, 168, 192, 216, and 240) respectively.

Nitrogen source: Various nitrogen sources, like ammonium chloride, ammonium sulphate, ammonium nitrate, yeast extract, peptone, etc. were used and examined for their effect on enzyme production by replacing tryptone in the production medium.

Carbon sources: The effect of different substrates as carbon source like rice bran, sugarcane bagasse, saw dust, coconut husk, pineapple peel, grasses were tested for their effect on enzyme activity by isolated culture.

Bioethanol production: The best isolate on the basis of optimization is allowed to grow in CMC broth with natural carbon sources i.e., replacing carboxymethyl cellulose from media. Media contained natural carbon substrate for production of cellulolytic enzyme and to initiate saccharification process (Satheesh et al., 2009). After proper incubation (with mixing at 100 rpm) the culture broth was conditioned for coculturing of *Saccharomyces cerevisae* by addition of filter sterilized salt solution (Ameh et al., 1989). The simultaneous saccharification and fermentation was carried out at 27°C for 5 days in a stationary condition (Lenziou et al.,1994 ; Eklund and Zacchi, 1995). At the end of incubation, the culture broth was tested for alcohol production.

Test for Bioethanol production (qualitative analysis)

Reducing sugar test of culture broth: The total reducing sugar content of the broth was determined by DNS method (Miller 1959) with glucose as standard. For this, the sugar content was determined initially, before addition of *Saccharomyces cerevisae* and also after 5 days of incubation.

Other tests include esterification test, and Iodoform test:

Iodoform test: For this a few ml of culture broth and 1% iodine solution was taken in a test tube. In this dil. Sodium hydroxide was added until brown colour of iodine was discharged. The test tube was gently warmed on a water bath. Yellow precipitate indicated the presence of ethanol.

Esterification test: A few ml of culture broth and 1 ml of glacial acetic acid was added followed by addition of 2-3 drops of conc. H_2SO_4. Then the mixture was heated in a water bath for 10 minutes. Fruity smell indicated the presence of ethanol.

Result and discussion:

Isolation: The 6 isolates (CB1, CB2, CB3, CB4, CB5, and CB6) were obtained from soil sample. These isolates were maintained for screening.

Screening of isolate for cellulose degradation: A total of six isolates were obtained from the soil. The isolates were screened for their cellulase producing ability using CMC as a source of carbon and congo red to check clear zone produced by the hydrolysis of cellulose. Only three isolates were able to grow on CMC media (CB1, CB2, and CB5). The large clear zone was obtained from CB5 isolate. Since the sole carbon source in CMC media was carboxymethylcellulose, the clear zone in the medium indicated cellulose degradation by the isolates. The clear zone around the colonies shows the production of cellulase enzyme by the bacterial culture which degrade the cellulose present in the medium.

Similar methods were used for the selection of best cellulase producing bacteria on the basis of clear zone diameter (Gautam et al., 2012, Ahmad et al., 2013 and Rasul et al. 2015). Cellulase producing *Bacillus* sp., isolated from soil and waste (molasses) of sugar industry, showed a zone of 9.5 mm on congo red plate (Rasul et al., 2015)

Enzyme production and assay: By the help of standard graph of glucose we estimated the concentration of glucose by crude enzyme which was obtained by isolated bacterial culture in CMC broth followed by centrifugation and optical density at 540nm as tabulated in table 1.

By estimating with standard graph of glucose we conclude that amount of glucose produced was 20 µg/ml. Therefore, by comparing the qualitative (clear zone) and quantitative (amount of glucose released) result we can say that the isolate CB5 was efficient cellulose degraders.

Characterization of the isolate:

The potential isolate (CB5) was identified on the basis of cultural and morphological characteristics.

Cultural characteristics: The bacterial isolate showed the following characteristics, such as, white coloured, regular, slimy and elevated colony on the agar media as tabulated in Table 2.

Morphological characteristics: The Gram's staining of the selected isolate was done and then observed under microscope at 40X. The isolated bacteria was Gram positive, Streptobacillus. As recorded in table 3.

Optimization of different parameters:

For optimization both growth and enzyme production were taken into consideration.

pH: The bacterial isolate was allowed to grow in media of different pH ranging from 4.5 to 7.5. Maximum enzyme activity and growth was observed in medium of pH 4.5 as shown in fig (1). With the help of a standard graph of glucose, we estimated that the conc. of glucose at pH 4.5 was estimated to be 74 µg/ml.

The highest cellulase production was found with Bacillus subtilis at p^H 6 (Gautam, 2012).

Temperature: The maximum enzyme activity was obtained at 45°C which was slightly reduced at 55°C. The increase in temperature, above the optimum values, results in loss of enzyme activity due to thermal denaturation of enzymes demonstrated in fig (2). With the help of the standard graph of glucose we state that the concentration of glucose at temperature 45°C was 40 µg/ml.

The highest cellulase production was found with Bacillus subtilis at 50°C (R.Gautam, 2012).

Nitrogen substrate: Production of extracellular cellulase has been shown to be sensitive by different nitrogen sources. The effect of nitrogen sources was studied in the growth medium, where tryptone was replaced by different nitrogen sources such as NH_4Cl, $NaNO_3$, yeast extract and peptone.

Among the various nitrogen sources tested, peptone was found to be the best nitrogen source for cellulase production for the isolated bacterial culture as in fig (3).

The highest cellulase production was found with Bacillus subtilis by utilizing peptone as nitrogen source (Bai et al., 2012)

Incubation period: Enzyme activity (cellulase) was recorded at different incubation periods i.e., 24 hours to 240 hours. As seen from fig (4) shown below, enzyme activity was maximum obtained at 216 hours of incubation and decreased above this due to depletion of nutrients or accumulation of other byproducts.

The highest cellulase production was found with Bacillus subtilis at the incubation period of 72 hours (Gautam, 2012).

Carbon sources: Different natural carbon sources such as coconut, pineapple, grasses, saw dust, sugar cane, rice bran were used instead of CMC to estimate their effect on cellulase production. The maximum activity was found with pineapple peel, coconut, grasses, saw dust and so on, as in fig (5).

Test for Bioethanol production (qualitative analysis)

Reducing sugar test of culture broth: Glucose concentration before and after addition of yeast. As we see, the glucose concentration was decreased after incubation with yeast culture (fig 6). So we can say that fermentation can take place.

Iodoform test: Yellow precipitate was obtained.

Esterification test: Fruity smell was observed.

Result was tabulated in table (4) shown below.

Conclusion:

This study provides the evidences for the production and optimization of cellulase production by isolated culture.

Out of the 6 isolates, three strains were screened for cellulase production using CMC media, of which one was selected for optimization on the basis of Congo red test and enzyme assay. The present study proved that peptone is a good nitrogen source for maximum production of cellulase enzyme. Natural substrate such as pineapple peel, coconut husk, saw dust, rice bran etc. are good carbon sources. Different optimization factors, such as, (pH, temperature, incubation period) were checked and it was observed that the best cellulase production was obtained at pH 4.5, at 45°C for 216 hours of incubation with peptone as nitrogen sources. This study is novel as it demonstrated efficient bioethanol production by culture strain through simultaneous saccharification and fermentation.

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Table 1. Optical density of crude enzyme

Isolate	Volume of crude enzyme taken in ml	Volume of buffer in ml	Volume of DNS reagent in ml	Absorbance at 540 nm
CB5	0.5	1	3	0.273

Table 2. Cultural characteristics of isolated bacterial colony

Isolate	Colour	Texture	Margin	Elevation
CB5	White	Slimy	Regular	convex

Table 3. Morphological characteristics of isolated bacterial colony

Isolate	Gram's reaction	Shape
CB5	Gram positive	*Streptobacillus*

Table 4. Result of sample (substrate)

Sample	Iodoform test	Esterification test
Pineapple	+	+
Coconut	+	Slightly positive
Grasses	+	Slightly positive
Saw dust	+	+

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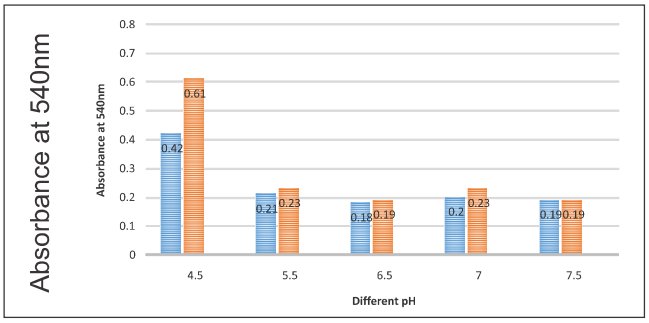

Fig. 1. Effect of pH (without culture and with culture)

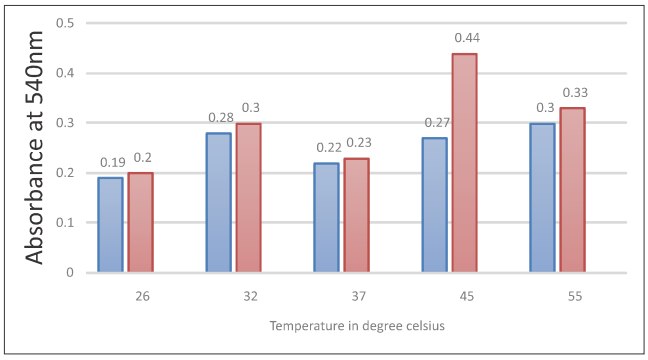

Fig. 2. Effect of temperature (without culture and with culture)

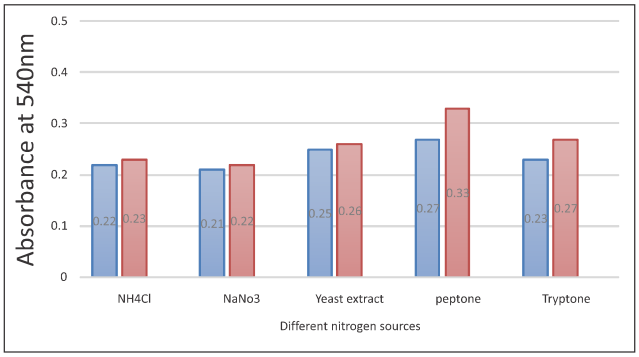

Fig. 3. Effect of nitrogen substrate (without culture and with culture)

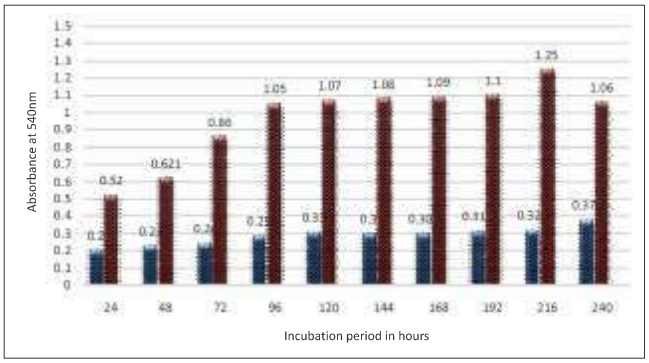

Fig. 4. Effect of incubation time (without culture and with culture)

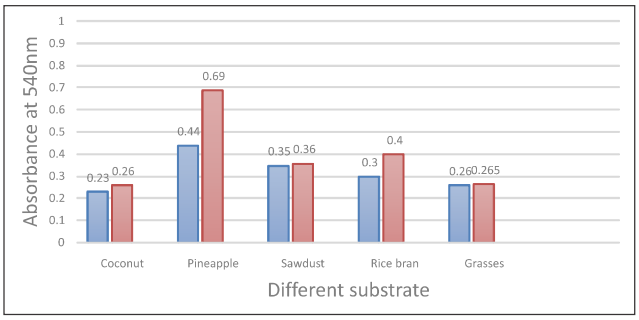

Fig 5. Effect of different substrate (without culture and with culture)

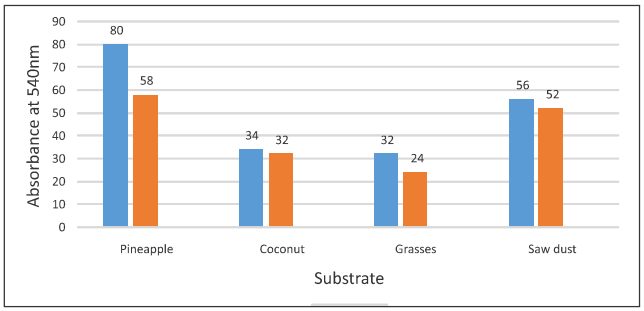

Fig. 6. Graph showing glucose conc. in µg/ml before and after addition of yeast culture

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