OPTIMIZATION OF CELLULASE PRODUCTION BY *Acinetobacter junii* ISOLATED FROM SOIL

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Abstract

Cellulose is the initial product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere. This enzyme is produced by several microorganisms, commonly by bacteria fungi and actiomycetes. The main objective of the study is to explore easy and cost effective method to produce the cellulase enzyme by using sugar cane bagasse, ragistraw and rice straw as substrate, which is an agro waste. Cellulase-producing bacteria were isolated from soil. Among the isolates, three strains showing maximum activity for purified and sub cultured on Carboxymethyl Cellulose (CMC) agar plates. Among the three strains, one strain was given maximum enzyme activity assay was measured by Dinitro Salicylic Acid (DNS) Method. The isolate were identified as *Acinetobacter junii* by 16S rRNA gene sequence analysis. Optimization of the fermentation medium for maximum cellulase production was carried out *via* submerged fermentation (SmF) using sugarcane bagasse, rice straw and ragi straw as substrates was studied. The highest production of cellulase was obtained with *Acinetobacter junii* when 3.0g sugarcane bagasse at 40°C, pH 7.5, 2% Inoculum level in 48 hrs.

Keywords: Cellulose, *Acinetobacter junii*, Agro waste, Submerged fermentation.

INTRODUCTION

Cellulases catalyze the hydrolysis of cellulose. Cellulose is the common organic compound on earth. It is basically the structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Plants are the most common source of renewable energy on the earth (Shankar *et al*., 2011). Cellulose is crystalline structure nature represents a big challenge for enzymatic hydrolysis. These crystals are sometimes so tight that neither water nor enzyme can penetrate them; only exogluconase, a subgroup of cellulase that attacks the terminal glucosidic bond, is effective in degrading it. The inability of water to penetrate cellulose also explains that crystalline cellulose is insoluble. The natural consequence in this difference of crystalline structure in the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose. Cellulases are generally of three types: endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β D glucosidase (EC 3.2.1.21) (Sreedevi *et al.,* 2013). Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic organisms and optimal conditions for catalytic activity by production of enzymes (Pérez *et al.,* 2002). Cellulases are inducible enzymes synthesized by microorganisms including fungi, bacteria and

actinomycetes during their growth on cellulosic materials. Filamentous fungi are the major source of cellulases and hemicellulases (Baldrian and Valásková 2008) but the production costs and generation is very high. Bacteria which have high growth rate and short generation time in compared to fungi have good potential to be used in cellulase production (Amore *et al.,* 2013). The potential cellulase producing bacteria are *Cellulomonas* species, *Bacillus* specics, *Ruminococcus albus, and Pseudomonas cellulose*. (Sonia Sethi *et al.,* 2013).

Complete bioconversion of cellulosic materials largely depends on the nature of cellulose, sources of cellulolytic organism and optimal conditions for catalytic activity and production of enzymes (Alam *et al.,* 2004). The present research was planned to screen the, bacterial population present in agricultural soil Dharmapuri. Tamilnadu and also identify the potent strains for cellulase production were screened and identified based on the phenotypic characteristics and 16S rRNA gene sequence analysis. Furthermore, we are also screened potential bacterial strains amoing the strain that gives maximum cellulase production. Different production parameters such as carbon sources, incubation period, temperature and pH optimized for enhanced cellulase production. There are several reports describing use of agro waste for the production of cellulose such as sugar cane bagasse, wheat bran and rice bran as substrate (Singhania *et al*., 2010). Compared to solid-state fermentation (SSF), and submerged fermentation (SmF) is widely used in industries since it is easy to operate.

MATERIALS AND METHOD

Sample collection

The soil samples were collected from different locations like paddy field in Namakkal, Tamilnadu. The soil samples were collected in a sterile plastic bag and then samples were immediately transported into the laboratory and processed and the remaining samples were stored in a refrigerator at 4ºC for further studies.

Screening and Isolation of Cellulase producing microbes

Collected samples were gently crushed and used for isolation by serial dilution technique. Cellulolytic microbes were isolated from the samples by using Carboxy Methyl cellulose (CMC) (Arusha *et al.,* 2016) medium was used for the isolation of cellulolytic bacteria from different samples and control was maintained. Techniques such as serial dilution, spread plate and streak plate methods were used for isolation and purification. Dilutions of 10^{-6} to 10^{-9} were used for bacterial isolation from soil samples and 10^{-4} to 10^{-8} from other samples. Serially diluted samples were spread (0.1 ml) on CMC plates and plates were incubated at 37°C for 24 hrs to 48 hrs. After incubation to visualize the hydrolysis zone the plates were flooded with

an aqueous solution of 0.1% Congo red for 15min and washed with 1MNaCl (Sonia Sethi *et al.*, 2016) To indicate the cellulose activity of the organisms, diameter of the clear zone around colonies.

Identification of the isolated microorganisms

The isolated organisms were identified by morphological examination and biochemical characterizations. Morphological test, Gram's staining, Endospore Staining, Motility test. Biochemical test Indole test, methyl red test, citrate utilization test, catalase test, oxidase test, and nitrate reduction test, carbohydrate (Arabinose, Glucose, Galactose, Fructose, Maltose, Lactose, Mannitol and Sucrose) fermentation test by standards methods (Buchanan *et al.*, 1974).

Phylogenic Analysis.

The partial 16S rRNA sequences was retrieved on NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLAST tool.

Maintenance of Bacterial Isolate:

The isolated bacterial culture was maintained in Nutrient Agar slants and stored at 4ºC to 7ºC for future use. Sub culture was performed every ten days interval.

Production Media:

The production medium containing, CMC (carboxymethyl cellulose) 1g, MgSo4 0.1g, Cacl2 0.1g, Fecl3 0.02g, K2HPo4 0.1g, Peptone 0.07g, broth was prepared in 100 ml conical flask and pH was adjusted to 7.0. The broth was sterilized and allowed to cool. Following cooling the medium was inoculated with a loop full of selected isolate. Then flask was incubated at 37ºC for 48 hours. After incubation the extra cellular enzyme produced in the culture medium was extracted by centrifugation. The crude enzyme preparation thus obtained was assayed for Dinitro Salicylic Acid (DNS) method.

Cellulase enzyme assay:

Cellulase activity was measured by Dinitro Salicylic Acid (DNS) Method (Miller, 1959). Add 3 ml of DNS reagent to 3 ml of enzyme sample in a lightly capped test tube. Heat the mixture at 90º C in water bath for 5-15 minutes to develop the red-brown colour. Add 1 ml of a 40% potassium sodium tartrate solution to stabilize the colour. After cooling to room temperature in a cold water bath, note the absorbance with a spectrophotometer at 575 nm.

Optimization of Cellulase Enzyme Production by Isolated Bacteria

Production and optimization of Cellulase from the isolates by using different physicochemical characters viz., incubation period, temperature, pH, carbon source, nitrogen source and inoculums level were studied to optimize the enzyme production (Kaniz Fatema and Manchur 2015).

Substrates for Cellulase production

Agricultural by products rich in cellulosic and other nutrients can be exploited as cheap raw material for the production of industrially important enzymes. Various agro-wastes such as sugarcane bags, Rice straw and Ragi straw which are cheaper can be used as substrates and thus reduce the cost of enzyme production (Lee *et al*., 2008).

Different fermentation techniques used for Cellulase enzyme Production.

Cellulase production was carried out by two different fermentation methods, such as solid state fermentation (SSF) and submerged fermentation (SmF).

RESULT AND DISCUSSION

Isolation and Screening of the Cellulase Producing Bacteria

Cellulolytic bacteria are well known agents of decomposition of organic matter in general and of cellulosic substrate in particular as reported by (Lynd *et al*., 2002). Bacteria can utilize wide range of cellulosic waste materials; therefore interest in the search for cellulase producing novel bacterial species is increasing the energy in earth. The present study was carried out with an aim of isolating, screening and identification of efficient cellulase producing bacteria from paddy field soil.

The cellulase producing bacteria were isolated from paddy field soil samples by serial dilution method and spread plating on CMC agar. The isolates were named as *Acinetobacter junii*. Carboxymethyl cellulose (CMC agar is a selective media for growth of the cellulolytic microorganisms because cellulase producing organisms can only utilize cellulose as the carbon source. The screenings of the cellulolytic bacterial isolates were performed based on the clearing zone around the colony on the CMC agar medium.

The appearance of clearing zone around the colony after the addition of 0.1% congo red solution was strong evidence that the bacteria produced cellulase in order to degrade cellulose. The bacterial culture was grown on CMC agar. The clear zone around the colony bacterial strains were identified as efficient cellulase

producing bacteria and the isolate *Acinetobacter junii* has given highest clear zone diameter **(Plate -1)** and its initial identification was done by gram staining, colony morphology and biochemical tests. The isolate *Acinetobacter junii* has been used for further studies in the enzyme production and their ability to degrade cellulose.

4.2 Morphological and Biochemical Characteristics of *Acinetobacter junii*

The isolate *Acinetobacter junii* purified by repeated sub culturing on the nutrient agar medium at regular intervals and stored at 4ºC. The isolates were identified based on the morphological and biochemical characteristics (**Table No: 1**).

Table No: 1

Biochemical Characters for *Acinetobacter junii*

Biochemical Test	**Results**
Indole test	Negative
Methyl Red test	Negative
Voges Proskar test	Negative
Citrate utilization test	Positive
Urease test	Negative
Triple Sugar Iron agar test	K/A
Oxidase test	Negative
Catalase test	Positive
Carbohydrate test:	
Glucose	AG
Lactose	Negative
Sucrose	Negative
Manitol	Negative
Maltose	Negative
Motility test	Non Motile

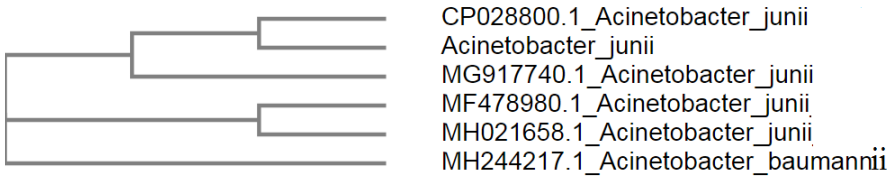
K/A- Alkaline Slant Acid butt, AG-Acid Gas, A-Acid

The morphology of isolates is milk creamy white, flat rough colonies with irregular edges and spreading rapidly on the surface of nutrient agar medium. They are gram negative coccobacilli singles with spares. Which are the characteristics biochemical properties (**Table No: 1)** of cellulolytic bacteria such as *Acinetobacter junii.*

Phenotypic Characteristics.

The 16S rRNA gene sequencing was carried out by using the primers, 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3'). The PCR product was purified and sequenced as described previously. The nucleotide sequence of isolates was aligned with selected sequences obtained from GenBank by using version MK MK249654.

Phylogenic tree

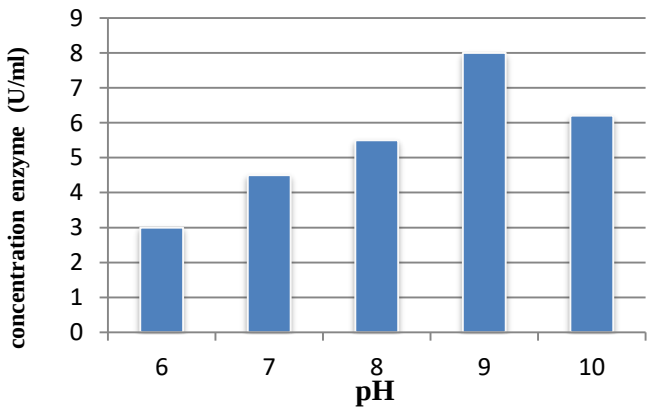


PRODUCTION PARAMETERS FOR CELLULASE ENZYME

Effect of pH on Cellulase Production

The pH for enzyme production by *Acinetobacter junii* is detected in the production medium. The *Acinetobacter junii* isolate selected from the soil sample one of them was founded to at pH values beyond 6, 7, 8, 9 and 10 that strain has been used in the present study for cellulase production. From the experiment of present the study the optimum pH 9, the maximum enzyme production was found to be *Acinetobacter junii* 8.0 (U/ml) **Figure: 1.**

Fig-1: Effect of pH on enyme production by *Acinetobacter junii*

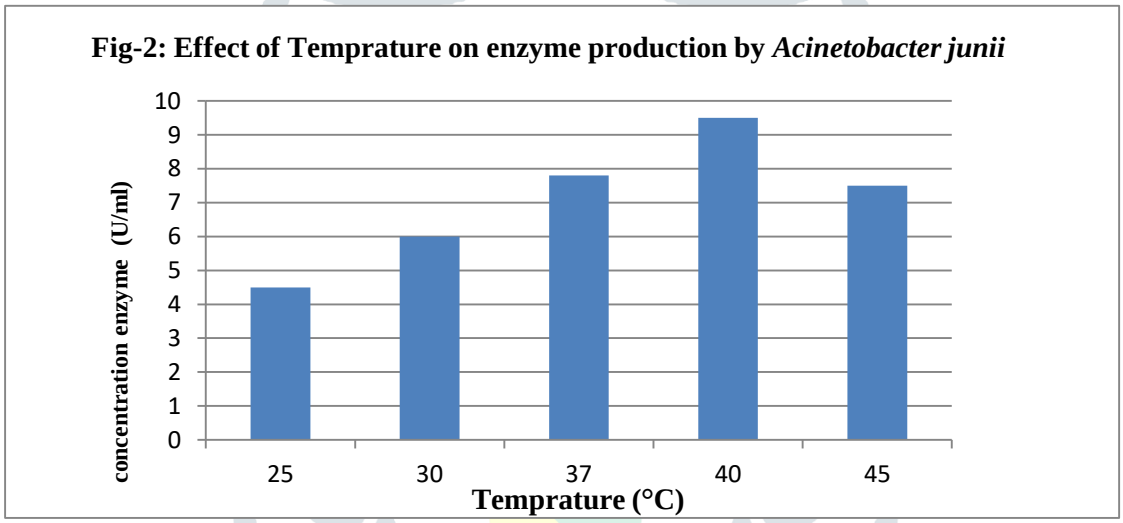


It might be due to the depletion of nutrients in the medium which stressed the bacterial physiology resulting in the inactivation of secretary machinery of the enzymes (Araffin, 2006) most of the *Bacillus subtilis* are maintaining log phase from 3 to 12 hrs of its growth. This variation of log phase timing is based on the temperature, pH and nutrients present in the medium and the cultural condition of the organism (Yang *et al*., 1995).

Effect of Temperature on Cellulase Production

The optimum temperature for enzyme production with selected isolate of *Acinetobacter junii* was found to be 25, 30, 37, 40, 45°C**.** When the optimum temperature of, maximum enzyme production found at 40°C by *Acinetobacter junii* 9.5 (U/ml). **Figure: 2**

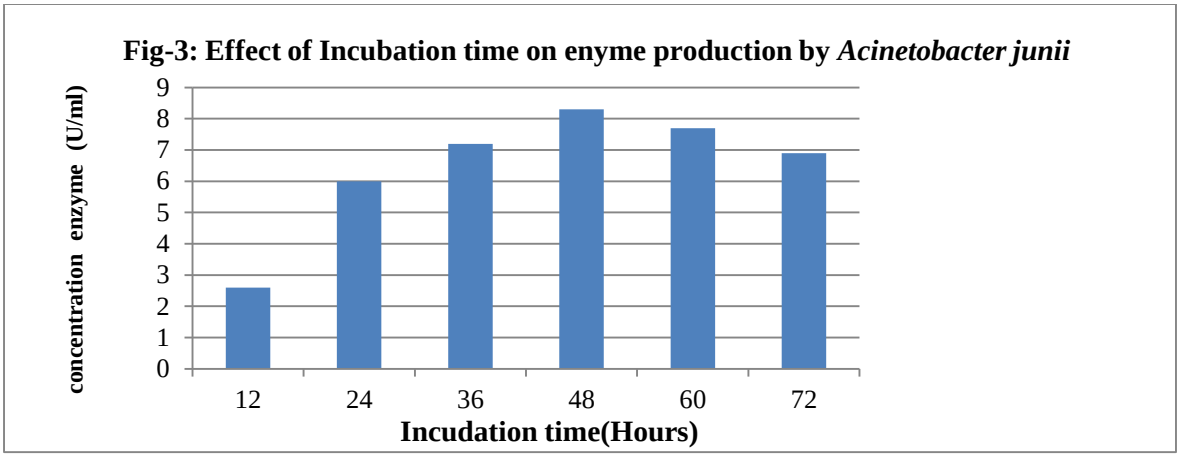
Fig-2: Effect of Temprature on enzyme production by *Acinetobacter junii*



Temperature is also an important factor that influence the cellulase yield maximum enzyme production by *Acinetobacter junii* was found at 40ºC. Many workers have reported different temperatures for maximum cellulase production either in flask (or) in fermentation studies using *Bacillus subtilis* suggesting that the optimal temperature. For cellulase enzyme production depends on the strain variation of the microorganisms. (Immanuel *et al.,* 2006). The maximum cellulase enzyme activity was recorded in *Cellulomonas*, *Bacillus* and *Micrococcus species* at 40ºC.

Effect of Incubation time on Cellulase production

When the time is taken for the maximum enzyme production was studied, maximum yield was found to be at 48 hours of incubation for enzyme produced by *Acinetobacter junii* 8.3(U/ml) **Figure: 3**



The enzyme production is depends on the depletion of nutrients in the medium which stressed the bacterial physiology resulting in the inactivation of secretary machinery of the enzymes (Araffin, 2006) most of the Baci*llus subtilis* are maintaining log phase from 3 to 12 hrs of its growth. This variation of different phase timing is based on the nutrients present in the medium and the cultural condition of the organism (Yang *et al*., 1995).

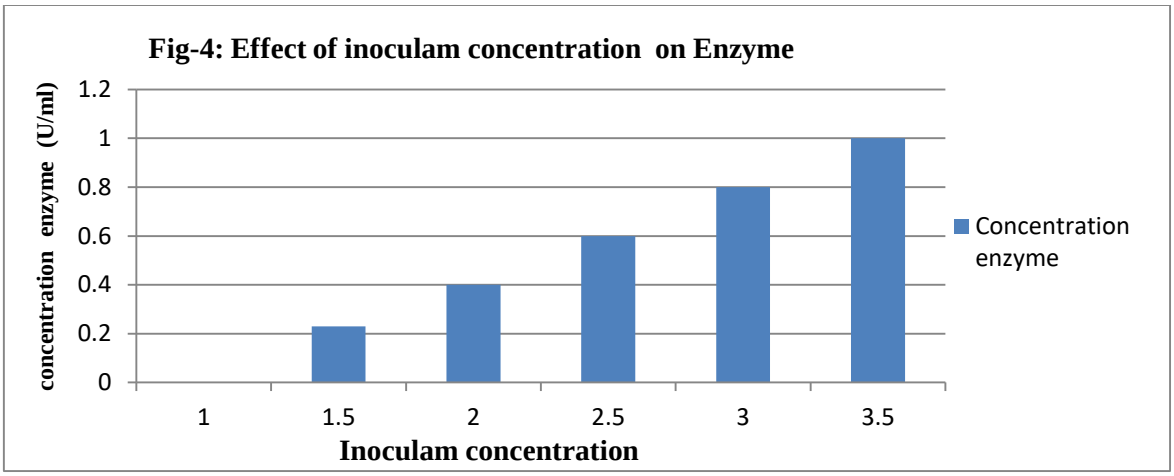
Effect of nitrogen source on Cellulase production

The Nitrogen source plays an important role in cellulase enzyme production. It effect on enzyme production by *Acinetobacter junii* was studied by supplementing different nitrogen sources such as peptone, tryptone, beef extract, malt extract and yeast extract into production media. Different nitrogen tested individually at the concentration of 0.5% in production media and is incubated at 40ºC for 48hrs.

The maximum cellulase enzyme production observed in media supplemented with malt extract (0.81U/ml) as nitrogen source. The organic nitrogen sources were more suitable for optimizing the cellulase production by Baci*llus subtilis*, than inorganic sources (Ray *et al*., 2007).

Effect of Inoculum level on Cellulase Production

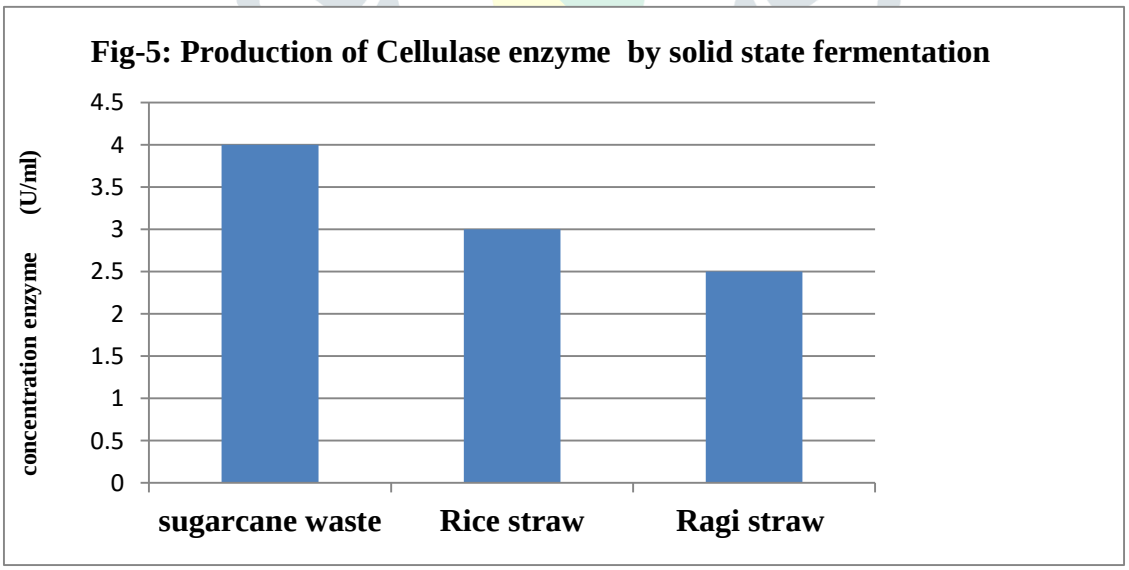
The effect of inoculums level on enzyme production was studied by inoculating different concentration of inoculums ranges from 1% to 3.5% in CMC broth. The different concentration of initial inoculums plays a critical role in enzyme yield in production media. The media was inoculated with different concentration of inoculums and incubated at 40ºC for 72 hrs. The optimum enzyme production observed in inoculums concentration ranges from 1% to 3.5% of inoculums **(fig 4).** The maximum production obtained in 3.5% (0.887 ± 0.02U/ml) followed by 2.5 and 1.5% (0.43 ± 0.02U/ml)



Solid State Fermentation

Production of Cellulase enzyme was carried out with different substrates viz., sugar cane bagass, rice straw and ragi straw used under solid state fermentation. 5g of each substrate was hydrated with mineral solution and adjusted. The moisture content was adjusted to 60%. 1% to 3.5% inoculum (24 hrs grown bacterial culture and 1×10^6 / ml) was inoculated and incubated at room temperature 25°C to 45°C for 48 to 72 hrs for bacterial cultures. After incubation, 22 ml of saline was added in each flask and incubated in rotary shaker for 15 to 30 min at 120 rpm.

From these result, it has been observed that, sugarcane waste broth has a higher yield of enzyme from *Acinetobacter junii* 4 (U/ml) **Figure: 5.**



Conclusion

The present work was isolate and identified a high cellulase producing bacteria from soil. The isolated strain *Acinetobacter junii* produced maximum cellulase yield at pH 7.0 and 40 °C temperature on 48 h incubation period at sugarcane bagasse as a substrate during fermentation have been production of cellulase. A further study was progress in the purification and application of cellulase in different commercial fields.

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