

Research Article



Optimization of Cellulolytic Bacteria from Cellulose Waste Materials and its Activity

T. Vinotha, N. Uma Maheswari*

PG and Research Department of Microbiology, Sengamala Thaayar Educational Trust Women's College, Mannargudi, Tamilnadu, India.

*Corresponding author's E-mail: vino.mb08@gmail.com

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ABSTRACT

Cellulases are the group of hydrolytic enzymes and they are capable of degrading all types of cellulosic waste materials. Present study focuses on the isolation, screening and enzymatic hydrolysis cellulolytic bacteria from various agricultural wastes under submerged fermentation and optimization of pH, temperature, carbon and nitrogen sources for the activity of enzyme production was tested. The enzyme reaction was carried out by CMC (Carboxy methyl cellulose) and the production was analyzed individually by Miller's modified method of Dinitro-salicylic acid (DNS). The cellulase producing bacteria was identified as *Bacillus sp* from Tapioca leaf, *Pseudomonas aeruginosa* from sugarcane Bagassee, *Pseudomonas flourescens* from Bamboo leaf, *Cellulomonas sp* from Sweet lemon peel, *Micrococcus sp* from Tapioca shell, *Streptococcus sp* from Turmeric leaf and *Lactobacillus sp* from Groundnut shell etc are the isolates shown a good results. Seven different bacterial strains were isolated and screened for cellulase production in submerged fermentation process. Among these seven tested bacterial strains; *Bacillus sp* secretes maximum amount of cellulose production around 7.896(IU/ml) under a submerged fermentation. The optimum pH and temperature for the activity of enzyme was 7 and 50°C and acetate as carbon source and ammonium sulfate as nitrogen source respectively.

Keywords: Agricultural waste, Cellulases, Cellulase producing bacteria, CMC (carboxymethylcellulose).

INTRODUCTION

Cellulose is the most abundant biopolymer on earth and most dominating agricultural wastes. This cellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value added bio-products. It can be degraded by cellulose produced by cellulolytic bacteria.¹ Cellulose is biologically renewable resource abundantly found in agriculture waste. The cellulosic waste material can be hydrolyzed to glucose and other soluble sugars by using cellulase enzymes of bacteria and fungi. Cellulose can be degraded by an enzyme called cellulase. The reducing sugars obtained can be further used for the production of ethanol as biofuel.

Agricultural wastes include crop residue, animal excreta and crop-processing wastes, slashing generated in logging, sawdust formed in timber production, and wood products in forestry originated activities. Agricultural and industrial wastes are among the causes of environmental pollution. Their conversion into useful products may ameliorate the problems they cause. These wastes which include mainly leaves, straw, cereals, corncobs, etc in many countries, these materials are generally used as animal feeds. A huge amount of these materials are left on farmlands to be decomposed by microorganisms such as bacteria and fungi.² Cellulosic waste products like Groundnut shell sweet lime peel, tapioca shell, tapioca leaves, bamboo leaves, sugarcane bagassee, and turmeric leaf were used in this study for the enzyme production.

Application of enzymes in textile, food, detergent, leather and paper industries demands identification of highly stable enzymes active at extreme pH and temperature.³

Cellulase is used in the fermentation of biomass into biofuels, fibre modification and they are even used for pharmaceutical applications. Bacteria has high growth rate as compared fungi has good potential to be used in cellulase production.⁴ For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts.⁵ Industrially important enzymes have traditionally been obtained from submerged fermentation (SMF) because of the ease of handling and greater control of environmental factors such as temperature, pH carbon and nitrogen sources.⁶

Present study is aimed to isolate and screening of cellulase producing bacteria from agricultural waste products, which can produce more efficient cellulase enzymes under submerged fermentation.⁷ 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.

MATERIALS AND METHODS

Collection of sample

The waste samples were collected from agricultural field and waste lands of virudhunagar district, Sathyamangalam, Erode district, Tiruchirapalli, Tuticorin in South India. The samples are sugarcane bagassee, tapioca shell and leaves, orange shell, bamboo leaves, ground nut shell, turmeric leafs etc are the waste materials were used for the isolation of bacteria.⁸



Sampling done by taking all possible aseptic measures and was stored at 4°C.

Isolation of Cellulolytic Bacteria

Cellulolytic bacterial strains were isolated from the waste samples by using serial dilution and pour plate technique. The medium used for isolation of bacteria nutrient media contain peptone-0.5%, beef extract-0.3%, yeast extract-0.75%, NaCl-0.5%, Agar-1.5%, and pH-6.8. Bacterial colonies were purified by repeated streaking.⁸ The purified colonies were preserved at 4°C for further identification and screening for cellulase production.

Screening of Cellulolytic Bacteria

Pure cultures of bacterial isolates were individually transferred in nutrient agar with 1% of Carboxy Methyl Cellulose in agar plates. After incubation for 48 hours, nutrient with CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counter staining the screened plates.⁸ Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies have the largest clear zone were selected for identification and cellulase production in submerged system.

Identification of Cellulolytic Bacteria

Identification of cellulolytic bacteria was carried out by gram staining based on morphological characteristics and biochemical characteristics.⁸

Inoculum Development

Pure cultures of selected bacterial isolates were individually maintained on CMC supplemented minimal agar slants at 4°C, were used. Pure cultures of selected bacterial isolates were inoculated in broth medium containing 0.03 % MgSO₄, 0.2 % K₂HPO₄, 1 % glucose, 0.25 % (NH₄)₂SO₄ and 1 % peptone at pH-7 for 24h of fermentation period.⁸ After 24h of fermentation period these vegetative cells were used as inoculums source.

Submerged Fermentation

Submerged fermentation was carried out in 250ml Erlenmeyer flasks containing 100ml of fermentation medium. The composition of the medium contained the following g/l of distilled water KH₂ PO₄-0.2, (NH₄)₂ SO₄-0.14, Urea-0.03, MgSO₄-0.03, CaCl₂-0.03, FeSO₄-0.5, MnSO₄-0.16, ZnSO₄-0.14, CaCl₂-0.2, groundnut shell, sweet lime peel, tapioca shell, tapioca leaves, bamboo leaves, sugarcane bagasse, and turmeric leaf. The medium was sterilized by autoclaving at 121°C for 15 min. Each flask was inoculated with 1ml of the above said inoculums. The cultures were incubated on a rotary shaker 120rpm at 30°C for 72h.⁹

Estimation of Cellulase Activity

Cellulase activity was assayed using dinitrosalicylic acid (DNS) reagent by estimation of reducing sugars released from CMC solubilized in 0.05 M sodium Citrate buffer at

pH 8. The culture broth was centrifuged at 14000 × g for 10 min at 4°C and the clear supernatant served as crude enzyme source. Crude enzyme was added to 0.5 ml of 1 % CMC in 0.05 M sodium citrate buffer and incubated at 50°C for 30 min. After incubation, reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100°C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm⁸. Cellulase production was estimated by using glucose calibration curve. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1µmol of glucose per minute under standard assay conditions.

Effect of Carbon and Nitrogen Source on Cellulase Production

The optimized media were prepared using pH range such as 4, 5, 6, 7, and 8 respectively by adding 1% NaOH and concentrated HCl. Then the media were autoclaved. Later they were inoculated with broth culture and were placed in a shaker (150rpm) at 37°C for 2 days. Simultaneously, for both organism and substrates were assayed. The optimized media were prepared individually by using the substrates and autoclaved. Later it was inoculated with broth culture and was act at different temperatures 30, 35, 40, 45 and 50°C respectively. The effect of temperature on the production of cellulolytic enzyme was determined by growing the organisms at the above temperatures.¹⁰ Simultaneously for the organisms and both the substrates, separate assay was carried out.

RESULTS AND DISCUSSION

Bacteria are well known agents of decomposition of organic matter and of cellulosic substrate.¹¹ Habitats that are rich with cellulosic substrates are the best sources in which we can isolate cellulolytic microorganism's from Agricultural wastes, Decayed cellulosic waste, compost, soil, decayed fruit etc were selected as a source for obtaining desirable cellulase producing organisms, because these are rich source of diverse group of cellulolytic microorganisms.

In the present study, seven cellulolytic bacteria were isolated from cellulosic waste samples. These seven bacteria were screened for cellulase production in submerged fermentation process using a suitable medium. The seven isolates were obtained from the samples namely *Bacillus sp* from Tapioca leaf, *Pseudomonas aeruginosa* from Sugarcane Bagasse, *Pseudomonas fluorescens* from Bamboo leaf, *Cellulomonas sp* from Sweet lemon peel, *Micrococcus sp* from Tapioca shell, *Streptococcus sp* from Turmeric leaf and *Lactobacillus sp* from Groundnut shell was identified by using morphological and biochemical tests. Microscopic examinations of these isolates were shown in the Table 1.

These isolates were maintained in CMC agar slants to get a pure culture. Basal media for *Pseudomonas aeruginosa*, *Bacillus sp*, *Pseudomonas fluorescens*, *Cellulomonas sp*,



Micrococcus sp, *Streptococcus sp* and *Lactobacillus sp* was prepared and production of cellulase was confirmed by Dinitro salicylic acid (DNS). The cellulase producing *Bacillus sp*, *Pseudomonas fluorescens*, *Lactobacillus sp* and *Pseudomonas aeruginosa*, was identified with the help of well zone formation in the cellulose fermentation medium than the other three bacteria. The zone is formed due to the cellulolytic activity of the organisms, which cleaves the hemicellulose molecules are present in

the medium. *Bacillus sp*, which only showed a highest response for the cellulase production which was isolated from the Tapioca leaves which requires a pH-7 and temperature at 50°C and grows well in acetate as a source of carbon and ammonium sulfate as a source of nitrogen. Enzyme production was carried out by submerged fermentation secretes maximum amount of cellulases around 7.896(U/ml) when it was grown along with waste substrates.

Table 1: Staining and Biochemical characteristics of the bacterial isolates

Organism	Gram staining	Indole	MR	VP	Citrate	Glucose	Nitrate	Sucrose	Catalase	Oxidase	Motility
<i>Bacillus sp</i>	Gram Positive	-	+	+	+	-	+	+	+	-	+
<i>Micrococcus sp</i>	Gram positive	-	-	-	-	-	-	-	+	+	-
<i>Streptococcus sp</i>	Gram positive	-	+	-	-	-	+	-	+	-	-
<i>Pseudomonas aeruginosa</i>	Gram negative	+	+	+	-	+	+	-	+	+	+
<i>Pseudomonas fluorescens</i>	Gram negative	+	+	+	-	+	+	-	+	+	+
<i>Cellulomonas sp</i>	Gram positive	-	-	-	-	+	+	+	+	-	+
<i>Lactobacillus sp</i>	Gram positive	-	+	-	-	+	-	+	-	-	+

“+” Positive; “-” Negative

Estimation of cellulose activity-DNS method

Cellulolytic activity was carried out for measuring zone of hydrolysis on 1% CMC agar medium by Plate diffusion assay method. Fermentation was monitored day wise by estimating reducing sugars by DNS method. Enzyme activity was determined spectrophotometrically as adapted from the recommendations of the Commission of biotechnology.¹² The enzyme assay produced around 1.86 IU/ml shown in Table 2.

Table 2: Estimation of cellulolytic activity by DNS method

Organism	Enzyme activity (DNS method) value IU/ml/min
<i>Bacillus sp</i>	1.86 IU/ml
<i>Pseudomonas aeruginosa</i>	0.67 IU/ml
<i>Pseudomonas fluorescens</i>	0.11 IU/ml
<i>Cellulomonas sp</i>	0.14 IU/ml
<i>Micrococcus sp</i>	0.76 IU/ml
<i>Lactobacillus sp</i>	0.56 IU/ml
<i>Streptococcus sp</i>	0.39 IU/ml

Optimization of cultural and nutritional conditions

The cellulose producing *Streptococcus sp*, *Bacillus sp*, *Lactobacillus sp* and *Pseudomonas aeruginosa*, was identified with the help of well zone formed in the cellulose fermentation medium than the other three

bacteria.¹³ The zone is formed due to the cellulolytic activity of the organisms, which cleaves the hemicellulose molecules are present in the medium. The results revealed the maximum cellulase production were noted in *Bacillus sp* from tapioca leaf, *Pseudomonas aeruginosa* from sugarcane bagasse, *Streptococcus sp* from groundnut shell, *Lactobacillus sp* from turmeric leaf. The cellulase production is around 7.896 IU/ml shown in Table 3.

Table 3: Estimation of cellulase productivity by CMC

Organism	Cellulase productivity(IU/ml)
<i>Bacillus sp</i>	7.896±0.09(IU/ml)
<i>Pseudomonas aeruginosa</i>	6.786±0.07(IU/ml)
<i>Pseudomonas fluorescens</i>	5.286±0.06(IU/ml)
<i>Cellulomonas sp</i>	4.363±0.05(IU/ml)
<i>Micrococcus sp</i>	3.346±0.03(IU/ml)
<i>Lactobacillus sp</i>	3.250±0.03(IU/ml)
<i>Streptococcus sp</i>	3.211±0.02(IU/ml)

Optimization of Culture Conditions on cellulase activity

In order to determine all effects on cellulase production, the selected bacterial isolates were grown in CMC broth and incubated at various parameters. The influence of all factors on enzyme activity was determined by measuring the following conditions were tested such as pH (4, 5, 6, 7, and 8), temperatures (30, 35, 40, 45, 50, 55, 60) both



carbon and nitrogen sources have wide variety of impacts on the various organisms and enzyme production. Carbon and nitrogen sources have been replaced with various substances. Carbon sources such as glucose, lactose, maltose, acetate, sucrose. Nitrogen sources such as sodium nitrate, urea, ammonium phosphate, ammonium

sulfate. All factors influence on enzyme activity was determined by measuring cellulose activity. The optimum pH and temperature for the activity of enzyme was 7 and 50°C and acetate as carbon source of 2.10 ± 0.8 and ammonium sulfate as nitrogen source of 2.05 ± 0.5 respectively shown in the table 4 and table 5.¹⁴

Table 4: Cellulase productivity of bacterial isolates for carbon and nitrogen sources

Name of the Substrate	Result	Carbon source	Submerged fermentation (IU/ml)	Nitrogen Source (IU/ml)	Submerged fermentation (IU/ml)
Tapiocca Leaf	<i>Bacillus sp</i>	Acetate	2.10±0.8	Ammonium sulfate	2.05±0.5
Sugarcane Bagassee	<i>Pseudomonas aeruginosa</i>	Glucose	2.08±0.6	Sodium nitrate	2.03±0.3
Bamboo Leaf	<i>Pseudomonas fluorescens</i>	Maltose	2.02±0.2	Urea	1.89±0.88
Sweet Lemon Peel	<i>Cellulomonas sp</i>	sucrose	1.20±0.78	Urea	1.34±0.66
Tapocca Shell	<i>Micrococcus sp</i>	acetate	0.85±0.06	Ammonium phosphate	0.96±0.41
Turmeric Leaf	<i>Lactobacillus sp</i>	lactose	0.66±0.04	Ammonium sulphate	0.56±0.06
Groundnut Shell	<i>Streptococcus sp</i>	glucose	0.23±0.01	urea	0.13±0.03

Values are represented as mean "±" Standard deviation

Table 5: Effect of pH and Temperature

Organism	Optimum temperature	Optimum pH
<i>Bacillus sp</i>	50°C	7
<i>Pseudomonas aeruginosa</i>	37°C	6.6-7
<i>Streptococcus sp</i>	37°C	5.3
<i>Lactobacillus sp</i>	30°C	5.7-6
<i>Pseudomonas fluorescens</i>	25-30°C	8
<i>Micrococcus sp</i>	34°C	5.5-6
<i>Cellulomonas sp</i>	60°C	7

CONCLUSION

Finally the *Bacillus sp* showed a higher response for the cellulase production isolated from the Tapiocca leaves which requires a pH-7 and temperature at 50°C and grows well in the acetate as a source of carbon and ammonium sulfate as a source of nitrogen. We concluded that agro-land industrial waste residues are generally considered the best substrate for the production of cellulose enzymes under a submerged fermentation is no exception to that a number of substrate have employed for the cultivation of microorganisms to host of enzyme. The selection of substrate for the enzyme production in a submerged process, depends upon several factors mainly related with cost and availability of the substrate, and thus involves in screening of several agro wastes. Future work will progress in the bio-ethanol production by purified cellulase enzyme along with bacterial consortia isolated from the agricultural wastes.

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