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Research Article

Cellulase activity of Streptomyces glomeratus strain NVJ01

Optimization Of Cellulase Production By Various Species Isolated From Cellulose Rich Sites

Namrata Joshi* and Preeti Mahawar

Madhav University, "Madhav Hills" Opp. Banas River Bridge Toll, N.H.-27, P.O.– Bharja, Abu Road, Tehsil – Pindwara. PIN- 307 026, Dist.: Sirohi, Rajasthan. India

Abstract: Cellulase is group of enzymes responsible for degradation of cellulose, a plant polymer. Cellulase has vast applications in various industries and hence continuous research is going on for better source of cellulase. Among all the different types of sources, microorganisms are found to be a prominent source of enzymes. As microorganisms can be easily isolated, grown and maintained for longer duration, making them potential enzyme producers. Industrial scale production through various types of fermentation enables large scale production. These microorganisms can be easily isolated from garden and nursery soil, sawmills and other wood contaminated area. Here, a study was carried out to isolate potential microorganisms capable of cellulase production for industrial applications. Samples were collected from five different sites having probable higher population of cellulase producers. Media having carboxymethyl cellulose as sole carbon source was used for selective screening of cellulase producers. Based on the zone of clearance assay, six potential microorganisms were selected and optimized for cellulase production. Concentration of carbon sources, type of nitrogen source, temperature, time and pH were the key factors which were optimized in the study. Based on the results of the study, it was found that out of six isolated, 3 belongs to *Streptomyces* species, 2 belongs to *Pseudomonas* species and 1 belongs to *Jonesia*. *Streptomyces* and *Pseudomonas* are among the most common producers but here we have also got a less common producer from *Jonesia* family. Highest enzyme activity of around 4.1U/mL was found in the cellulase obtained from *Streptomyces glomeratus strain* NVJ01. Optimization has shown that, CMC concentration of 1.0% with ammonium nitrate gave highest production when incubated for 48 hrs. at 6.5 pH and 35°C.

Keywords : Cellulase, cellulose rich sites, *Streptomyces*, *Pseudomonas*, *Jonesia*

***Corresponding Author**

Namrata Joshi , Madhav University, "Madhav Hills" Opp. Banas River Bridge Toll,N.H.-27, P.O.– Bharja, Abu Road, Tehsil – Pindwara. PIN- 307 026,Dist.: Sirohi, Rajasthan. India

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1. INTRODUCTION

Cellulase is not a single enzyme but it is group of enzyme capable of degrading cellulose.[1-3] Cellulose is one of the most abundant and renewable sources obtained from plants. It remains always in high demands for animal feed, paper/pulp, manure and fuel. Effective utilization of this cellulose is a challenge as its microcrystalline structure cannot be degraded easily. It requires acid, alkali and/or heat treatment which make it less economic and environmental hazardous.[4,5] Enzymes can be used instead more efficiently without any adverse effect on environment. This enzyme is found in bacteria, fungi, protozoa, termites, earthworm and other insects. It breaks the 1,4-β-D glycosidic bond found in polymers like cellulose, hemicellulose, glucans, lichenin etc.[6-9] However, it cannot degrade the other common plant polysaccharide starch. There are main five kinds of cellulase exist in nature which are exocellulase, endocellulase, cellobiose, oxidative cellulase and cellulose phosphorylases. Out of them, exocellulase and endocellulase are in high demands in various industries.[10,11] Paper and pulp industry, fabric industry, laundry and detergent industry, agriculture industry, medical industry and energy sectors are among the leading industries.[10,12-15] For large scale production of this enzyme, cellulolytic microorganisms are preferred as they can be easily grown in larger quantity using various fermentation methods.[16-18,19] Optimization of environmental conditions enhances the production efficiency of microorganisms.[5,20,21] Majority of known microorganisms were isolated from common sources like garden soil and wood soil.[21,22] Cellulase isolated from each microorganism may have different chemical and enzymatic properties. Catalytic potential is considered to be one of the most vital property, where the ability of enzyme for degradation of cellulose is determined.[6] In the search for enzyme having highest catalytic potential, worldwide research is still going on. Maragathavalli *et al* (2015) had carried out a study where total of 73 species belonging to *Bacillus, Trichoderma, Aspergillus, Fusarium, Alternaria,* and *Penicillium* family were screened for cellulase production. When compared with bacteria, fungi have more potential to degrade and cellulose with the help of enzyme.[15] Zhang *et al* (2006) have given their valuable inputs for the screening and selection strategies for cellulase improvement. They have recommended using continuous culture technique with insoluble cellulosic substrate for higher activity and quantity of cellulase.[24] Here, a study was carried out to isolate cellulose degrading microorganisms from various soils samples. Selected microorganisms were further optimized for carbon source, nitrogen source, pH, temperature and time for optimum production of cellulase.

2. MATERIALS AND METHODS

2.1 *Isolation Of Microorganisms*

Soil samples (around 10 gms) were collected in a sterile container from garden and wood soils. For selective isolation, Bushnell Haas (BH) media with carboxy methyl cellulose (CMC) as a sole carbon source was used. Spread plate method, after serial dilution was performed for isolation of microorganisms. All the plates were allowed to incubated at 37°C for 24 hrs. Colonies having different colony morphology were screened and further purified on media containing plates.[20,25] Similarly, samples were also spread on a nutrient agar plate to determine the total cultivable microorganisms present in the sample.

2.2 *Screening Of Potential Microorganisms*

Screening for potential microorganisms was done using a very simple, yet effective method. In this method, zone of clearance by cellulase enzyme on CMC agar plate was taken into consideration to determine the efficiency. In the procedure, BH media with 1% CMC were spotted with 50µl of active culture. Plates were incubated for 24 hrs at 37°C. After incubation, plates were flooded with 1.0% Congo red for 20 minutes. Excess Congo red was poured off and plates were decolorized by 5M NaCl. Based on zone of clearance, potential microorganisms were further selected for the study.[20,26]

2.3 *Optimization Of Media And Environmental Parameters*

There are various components of the media which play vital role in production of enzyme. Among all, carbon and nitrogen sources are the preliminary components. In addition to that temperature of growth, time of incubation and pH of media was also been considered for optimum enzyme production.[5,20,22,27] In this study, CMC as a carbon source was optimized for its concentration from 0.5% to 2.5%. Total five different nitrogen sources namely ammonium nitrate, ammonium sulfate, ammonium chloride, ammonium molybdate and potassium nitrate were also optimized. For optimization of temperature, cultures were grown at 25^{O}C, 30^{O}C, 35^{O}C, 40^{O}C and 45 ^{O}C for 24 hrs on orbital shaker at 120 rpm. For optimization of pH, culture media were prepared with pH 4.5, 5.5, 6.5, 7.5 and 8.5 using sterile 1.0N HCl or 1.0N NaOH. For optimization of incubation time, cultures were grown for 120 hrs and aliquots were collected at regular interval of 24hrs. In all the experiments, 100 µl active culture having density equivalent to McFarland Constant 1 was added as starting inoculum. For determination of enzyme activity, dinitro salicylic acid (DNSA) assay was used. Folin Lowry method was performed to determine the concentration of proteins. [20,22]

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2.4 *Identification Of Microorganisms*

16S rRNA was used for identification of isolated microorganisms. Obtained sequences were submitted to NCBI database.[19]

3. STATISTICAL ANALYSIS

Obtained data were analyzed using SPSS 18.0 statistical software. Results were noted as mean ± standard deviation (Mean±SD)

4. RESULTS AND DISCUSSION

After 24 hrs of incubation, colonies obtained on the plates were counted. Obtained results were mentioned in the table below. (Table 1)

Table 1. Number of isolates obtained from various sample sites

Sr. No.	Type of Sample	No. of Colonies on Nutrient agar	No. of colonies on BH+ CMC	Percentage of cellulase producing Microbes
1	Garden Soil A	2.95×10^8	2.75×10^7	10.72%
2	Garden Soil B	2.75×10^8	2.50×10^7	11.00%
3	Garden Soil C	2.80×10^8	1.50×10^7	18.67%
4	Wood Soil	9.45×10^7	1.05×10^7	9.00%
5	Nursery Soil	2.80×10^8	2.75×10^7	10.18%

Results are represented as number of colonies per gram of samples along with percentage of cellulase producers.

Based on the results of isolation, it was observed that, in all kind of samples, around 12.0% to 13.0% microorganisms were capable of degrading cellulose. These microorganisms may be considered as an efficient cellulase producer. Microorganisms were further selected based on different colony morphology. However, it is not necessary, that all the obtained colonies produces significant amount of cellulase. To differentiate between efficient producers, zone of clearance study was carried out. Microorganisms capable of utilizing cellulose and able to produce cellulose, degrade CMC in the media which resulted into a clear zone upon staining and destaining with congo red and NaCl. Depending on the size of zone of clearance, the efficiency of cellulase production can be determined. Priyanka *et al* (2016) have found a very prominent zones in their study carried out for the fungi isolated from soil.[20] Similarly, Ram *et al* (2014) have also used the same method for easy screening of potential isolates.[23] Gohel *et al* (2014) have shown that congo red and iodine staining were the most efficient method for determination of zone of clearance.[26] Similar to this, many other studies have also used this technique for easy and fast screening of potential isolates. [28-30] Obtained results for zone of clearance are mentioned in the table below 2 and Figure 1 & 2.

Table 2. Results of zone of clearance of selected isolates

Sr. No.	Microbe ID/Plate ID	Zone of Clearance in mm (Mean±SD)	Sr. No	Microbe ID	Zone of Clearance in mm (Mean±SD)
1	1/A1	12.0 ± 1.0	13	13/A13	17.0 ± 2.0
2	2/A2	ND	14	14/A14	10.0 ± 1.0
3	3/A3	22.0 ± 2.0	15	15/A15	9.0 ± 1.0
4	4/A4	ND	16	16/A16	8.0 ± 2.0
5	5/A5	ND	17	1/B1	ND
6	6/A6	11.0 ± 1.0	18	2/B2	8.0 ± 1.0
7	7/A7	9.0 ± 1.0	19	3/B3	10.0 ±1.0
8	8/A8	13.0 ± 1.0	20	4/B4	10.0 ± 1.0
9	9/A9	ND	21	5/B5	ND
10	10/A10	10.0 ± 2.0	22	6/B6	9.0 ± 1.0
11	11/A11	ND	23	7/B7	18.0 ± 1.0
12	12/A12	ND	24	8/B8	ND

Zone of clearance was measured in millimeter (mm) and mentioned as Mean ± SD. ND=Not Detected

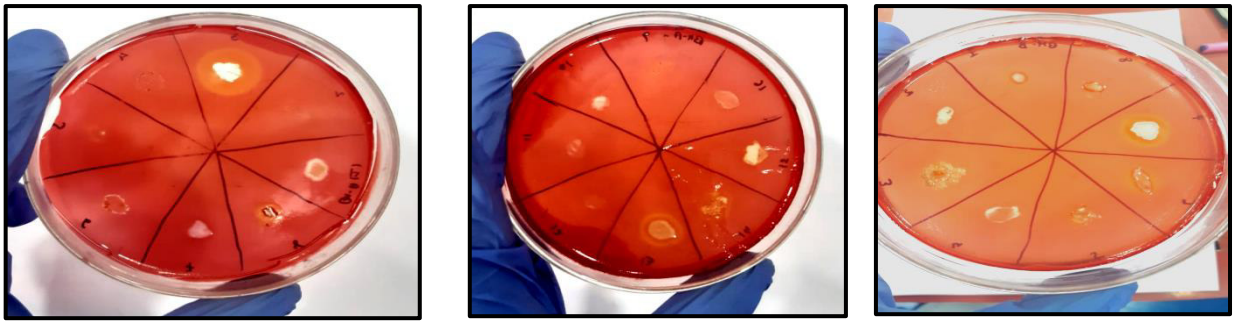

Fig 1. Zone of inhibition study for selected microorganisms. Zone of clearance were measured in millimeter against bright background

Based on the results of zone of clearance, it was found that, out of total 24 isolates, 15 were found to produce exocellulase. Highest zone of clearance was found with species no 3, followed by species no. 13 and 8. Least activity was seen in the isolate no 16. Based on the results six potential microorganisms namely species no 1, 3, 6, 7, 8 and 13 were selected further for the study. 16S rRNA Sequencing results have shown that, out of six microorganisms, 3 belongs to *Streptomyces* family, two belongs to *Pseudomonas* family and 1 belongs to *Jonesia* family. Table 3 provides a detailed information of identification. Many previous studies have shown that, few species of *Streptomyces, Pseudomonas, Bacilli, Penicillium, Trichoderma, Aspergillus* and *Clostridium* are potential cellulase producer.[7,8,10,14,20,21,3,28,31-34] Few of them have already in use for large scale production of enzyme for various applications. Schwarz (1989) have provided a detailed information of application of cellulases in agro-waste industries.[13] Kuhad *et al* (2011) and Maragathavalli *et al* (2015) have also provided details of applications of microbial cellulases for industrial purposes.[15] Even certain species were also found which can produce the enzyme under anaerobic conditions. Studies have shown that, the majority of the species belongs to *Clostridium* family [35,36]

Table 3. Results of identification

Sr. No.	Initial Isolate Number	Name of Microorganism	NCBI Accession Number
1	3	*Streptomyces glomeratus strain* NVJ01	MT953896
2	13	*Pseudomonas stutzeri strain* NVJ02	MT953897
3	1	*Pseudomonas alcaligenes strain* NVJ03	MT953898
4	8	*Streptomyces_sp.* NVJ04	MT953899
5	7	*Streptomyces minutiscleroticus strain* NVJ05	MT953900
6	6	*Jonesia denitrificans strain* NVJ06	MT953901

Table represents the identified species with their NCBI accession numbers.

4.1 Results Of Media Optimization And Environmental Conditions

When selected six microorganisms were optimized for CMC as carbon source, it was found that 1.0% CMC favour maximum growth of microorganisms with highest enzyme activity. CMC concentration of 1.5% also gave good growth, but it was found lesser then the 1.0% CMC (Figure 2). In most of the studies carried out for cellulase, CMC was preferred as a sole source of carbon. As it is an ideal substrate for cellulase, has good solubility and can be used in all solid, semisolid and liquid types of media. Other sources like waste papers, paper pulp, agriculture waste, molasses, baggase and fruit peels were also used in some studies. However, the production potential was found very less as compare to CMC.[13,16,] Fruit waste was also not found very suitable for cellulase for at industrial scale.[37] Coir and other agriculture waste can be recycled by cellulase but to be used as feedstock for large scale is not feasible.[38,39] However use of such feedstocks is a good way to recycle waste materials and to protect the environment. Among various nitrogen sources used for optimization, ammonium nitrate and ammonium sulfate were found very efficient(Figure 3). Results of optimization of temperature, time and pH have shown that these microorganisms can grow best at 35°C, 48 hrs with 6.5 pH. (Figure 4 to 6).

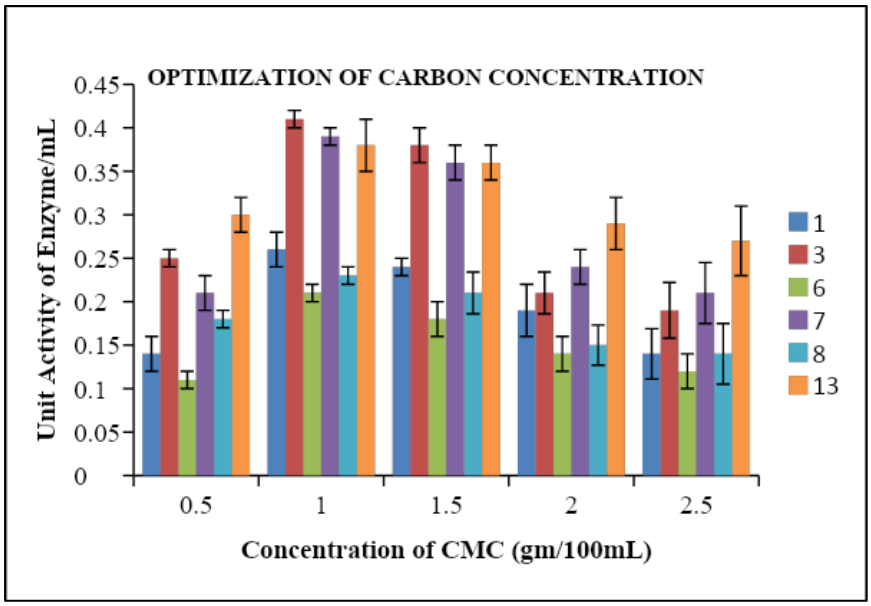

Fig 2. Optimization of CMC as carbon sources for extracellular cellulase production. Among different concentration of CMC, 1.0% and 1.5% concentration have given better production.

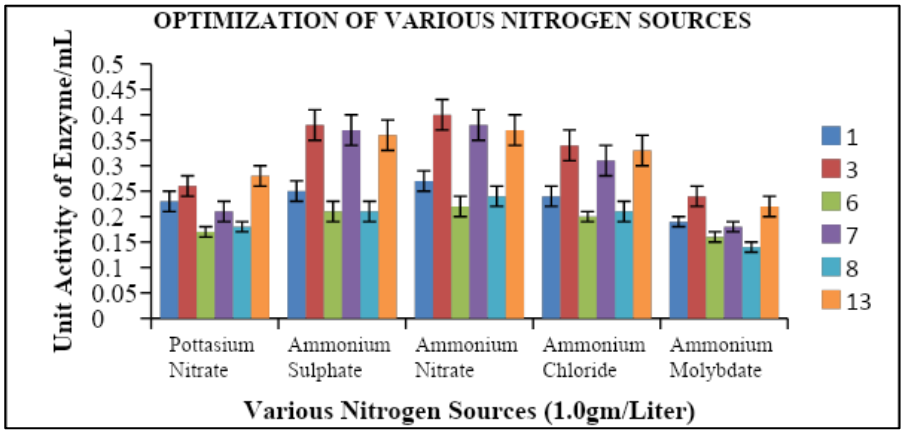

Fig 3. Optimization of nitrogen sources for extracellular cellulase production. Ammonium sulfate and ammonium nitrate have given higher production of enzymes as compare to other nitrogen source.

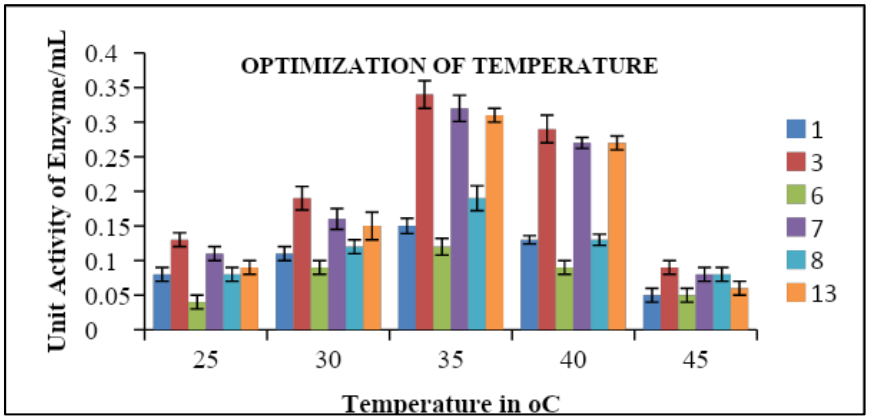

Fig 4. Optimization of temperature for extracellular cellulase production. All species have grown optimally at 35°C

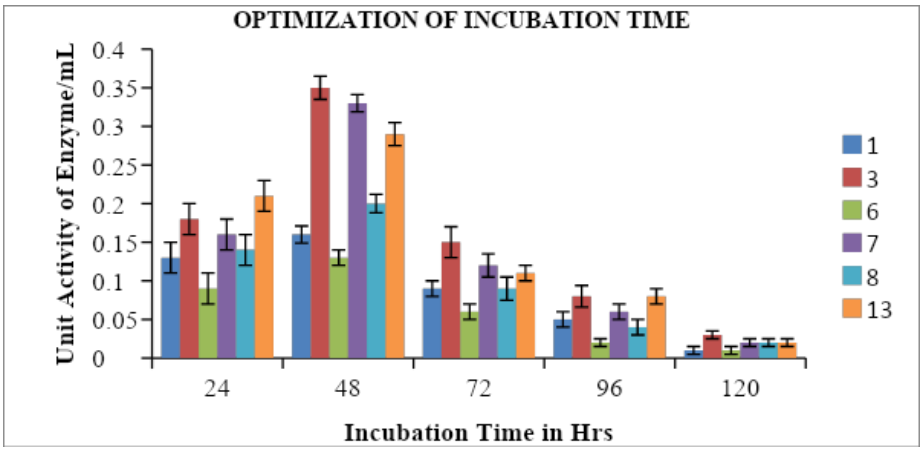

Fig 5. Optimization of incubation time for extracellular cellulase production. Within 48 hrs of incubation all the species have given maximum enzyme production.

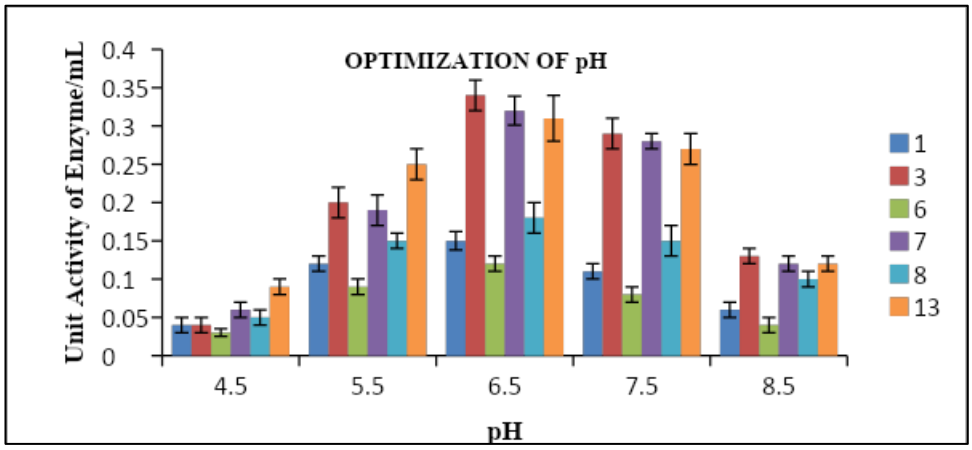

Fig 6. Optimization of pH for extracellular cellulase production. Near neutral pH at 6.5 and 7.5, all the microorganisms were able to grow. Highest activity of enzyme was found at 6.5

Since the samples were collected from the mesophilic site, it has favored maximum growth of microorganisms near the temperature 37°C. It was also seen that, at slightly elevated temperature (40°C), the growth of microorganisms reduced. Highest enzymatic activity of 3.4 U/mL was obtained in *Streptomyces glomeratus strain* NVJ01, which was reduced to 3.0 U/ml at 40°C. This suggests that temperature also plays a vital role in optimum production of enzyme. Similar to temperature, earlier studies have also proved that pH near to neutral favour maximum production of enzyme through optimum growth of microorganisms.[19,21,26,37] In batch type culture, cellulase production reaches higher between 36 to 48 hrs of inoculation.[16,] Predeep and Narasimha (2011) have used mutated fungi strain and found that production had started at maximum rate after 48 hrs and reached maximum at 7 days. [41]

5. CONCLUSION

Based on the entire study, it was concluded that potential cellulase producer can be easily isolated for various type of soil samples. In all the selected samples, around 10.0% species were capable of utilizing cellulose for growth. Concentration of 1% CMC is ideal for all the isolated strains. Ammonium salts are highly recommended for high production of enzyme. pH of media must be maintained near to neutral. For mesophilic isolates, range of 35°C-40°C is ideal. After optimization of all the essential parameters, maximum activity of cellulase activity was obtained from *Streptomyces glomeratus strain* NVJ01, whereas the least activity was observe in the cellulase obtained from *Jonesia denitrificans strain* NVJ06

6. ACKNOWLEDGMENT

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7. AUTHORS CONTRIBUTION STATEMENT

Dr. Preeti Mahawar has provided her guidance in planning and execution of entire study. She has also guided in interpretation of experimental results. Namrata Joshi has carried out the entire research work and also contributed in interpretation of results. Both the authors have contributed equally for preparation of manuscripts.

8. CONFLICT OF INTEREST

Authors have no conflict of interest

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