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Research Article

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Isolation of Potential Extracellular Cellulase Producer and Determination of Cellulase Production Efficiency with Various Raw Substrates

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Abstract: Cellulase is one of the important groups of enzyme for many industrial applications. It is applied in various processes of food industries, paper and pulp industry, fabric industry, medicine and pharma industry, agriculture industries etc. Microorganisms are among the most potential source for large scale production of this enzyme. The current study is aimed to isolate microorganisms which are capable of producing cellulase and to determine its efficiency for production of cellulase with different raw materials. For this study, bacteria microorganisms were isolated on CMC containing media. Screening was done using congo red (0.1%) and NaCl (1%) solution. Among various isolated species, *Bacillus amyloliquefaciens* was identified as the most potential strain and hence selected for further study, various raw materials which are rich in cellulose were used for cellulase production. These raw materials were sugarcane bagasse, paper pulp, molasses, orange peel, wheat bran, cassava waste, tea waste and agriculture waste. Cellulase activity was determined by using dinitrosalicylic acid (DNSA) method. Results of the study have shown that among all the raw materials, sugarcane bagasse, molasses and paper pulp were found as the most potential sources for enzyme production. DNSA have shown 0.97 IU/mL, 0.98IU/mL and 0.88 IU/mL of enzyme activity respectively for all the three raw materials. Results have also shown that agriculture waste alone is not found suitable for production of cellulase as it has shown only 0.32 IU/mL of enzyme activity. From the entire study, it was concluded that for large scale production of cellulase, sugarcane bagasse and molasses are the best sources using *Bacillus amyloliquefaciens*.

Keywords: *B.* amyloliquefaciens, cellulase, raw materials, sugarcane bagasse, paper pulp, DNSA

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1. INTRODUCTION

Cellulase is a group of enzymes which is responsible for the degradation of cellulose. This process is known as cellulolysis. Cellulase is mainly produced by fungi, bacteria and protozoa. [1-8] At a time more than one enzyme can involve in the degradation of cellulose and produce its monomers. Cellulose degradation is essential as it makes monomers available to various sites of plant for consumption and to provide energy in chemical reactions. [9-11] Chemical reaction involves the hydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose and cereal beta-D-glucans. Since cellulose is a very stable and non-reactive structure, its degradation is relatively difficult as compared to other polysaccharides like starch. According to the carbohydrate active enzymes database (CAZy) all cellulase are found in 12 GH families. [12,13] Main five types of cellulase are endocellulase (EC 3.2.1.4), exocellulase (EC 3.2.1.91), cellobiases (EC 3.2.1.21), oxidative cellulases and cellulose phosphorylases. Each of these five cellulases carries out a specific type of degradation reaction. Endocellulase as the name predicts breaks the internal bonds of cellulose, giving rise to a new open chain. Exo cellulose degrades from the ends of the chains resulting in the release of disaccharide or tetrasaccharide.[14] Cellobiases degrades disaccharide or tetrasaccharides into its monomer.[15-18] The other two enzymes are not directly involved in degradation but are highly active in oxidation and phosphorylation. All these five classes of enzymes are found in an organized form in bacteria which is known as cellulosomes.[19-21] Here, in this study isolated *B. amyloliquefanciens* was used for the study. *B. amyloliquefanciens* is a species belonging to phylum Firmicutes and family Bacillaceae. It can be isolated from varieties of sources. It is known for its enzyme production ability. It is also used as probiotic strains in certain food preparation. Previous studies have shown that *B. amyloliquefaciens* contains endo-1,4-beta-D-glucanase as key cellulase enzyme. In the present study, bacteria were isolated from cellulose rich samples. Bacteria were screened based on their cellulase enzyme production efficiency. Among all, the best strain was selected for further study. In the further study various parameters were optimized for optimum production of extracellular enzymes. Various raw materials were used as substrates for selection of the most suitable substrate for the cellulase production at commercial level.

2. MATERIAL AND METHOD

2.1 Isolation of Microorganisms

Selective isolation of cellulose producing microorganisms was carried out using carboxymethyl cellulose, CMC as a sole carbon source in the media. [22,23] The Cellulose rich sample was enriched in the Bushmell Haas (BH) media with 1% CMC and allowed incubating for 24hrs at 150rpm. After 24 hrs, culture was streaked on plates containing BH and CMC (1%). Plates were incubated at 37^{O}C till the visible colonies were seen. Certain colonies having different colony morphology were screened and stored on BH and CMC (1%) slant as pure culture for further study. [22,23]

2.2 Selection of potential cellulase producing strain

All the isolated strains were activated in BH and CMC (1%) broth and 50 µl of each strain was spotted on a plate with BH and CMC (1%) and incubated for 24 hrs at 37^{O}C. After 24hrs, plates were flooded with congo red (0.1%) and washed with 1N NaCl solution. [6,8,24] Zone of clearance was measured in mm and the strain having maximum zone of clearance was taken for further study.

2.3 Identification of strain

Selected stain was identified using 16s rRNA sequencing. Obtained sequence was compared with the NCBI gene databank for identification of strains.[21,27]

2.4 Determination of enzymatic activity using various raw substrates

Preparation of inoculum media: Strain was activated in media containing 0.25% KH2PO4, 0.2% Tryptone, 0.4% Na2HPO4, 0.02% MgSo4.7H2O, 0.0001% CaCl2 .2H2O, 0.0004%, FeSO4 .7H2O) pH=7 with 1% of raw material as carbon source. Sugarcane bagasse, paper pulp, cassava waste, tea waste, orange peel, molasses, wheat bran and agriculture waste were used as substrate for cellulase enzyme production Flask was incubated at 37^{O}C for 24 hrs or till the viable count reaches to 3 X 10^{6} cells/mL. [32,34,37]

2.5 Preparation of production media

Production media composition is also the same as inoculum media. Here instead of 1% of raw material 5% of raw material was added and flasks were allowed to incubate at 37^{O}C for 72 hrs at 150 rpm. [32,34]

2.6 Enzyme assay

After 72 hrs of incubation, culture was centrifuged at 10,000 rpm for 10 minutes at 4^{O}C. After centrifugation, the supernatant was collected and used as a crude enzyme. For enzymatic assay Dinitro salicylic method (DNSA) was done. In the assay, 1 mL of 0.05M citrate buffer containing 0.5ml of substrate (0.5%) was incubated with 1mL of crude enzyme and incubated for 10 minutes at 37^{O}C. Rate of reaction was determined based on release of glucose molecules upon hydrolysis of cellulose which was carried out using DNSA. [3,4,19,25] Folin Lowry was performed to determine the protein concentration in the crude enzyme. [22,23]

3. STATISTICAL ANALYSIS

Obtained data were statistically analyzed using SPSS 16.0. The data were represented as mean ± standard deviation (SD). One way ANOVA was performed to determine the significance of production of enzyme by various substrates. [32]

4. RESULTS AND DISCUSSION

Out of sixteen isolated species, only the best specie was subject of identification. When obtained 16s rRNA sequence was compared with the NCBI database it was found that the isolated species belongs to *B. amyloliquefaciens* (100.0% similarity) (Fig 1.) The sequence is submitted to NCBI and available with accession number MN081796.

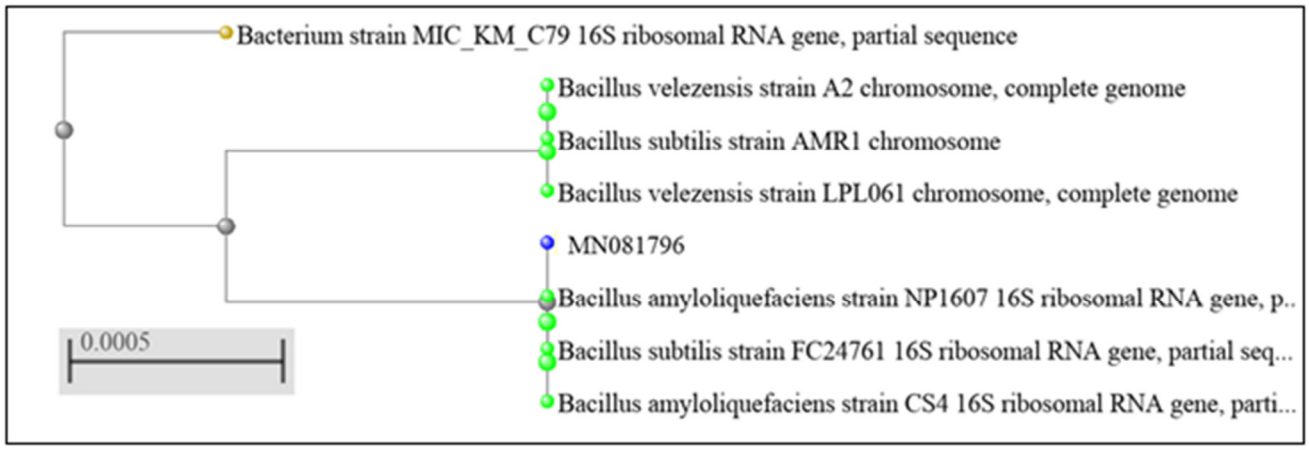

Fig1. Phylogenetic tree for isolated species MN081796

Based on the DNSA assay results, the following enzymatic activity was obtained for various raw materials. (Table 1) Based on the results obtained it was found that sugarcane bagasse and molasses are the most suitable sources for cellulase production followed by paper pulp. Whereas agriculture waste and tea waste are not suitable as the enzymatic activity is very low. (Figure 2)

Table 1. Activity of cellulase with difference raw substrates

Raw Substrate	Enzyme activity(Units/mg)
Sugarcane bagasse	0.97±0.12
Paper pulp	0.88±0.11
Cassava waste	0.55±0.06
Tea waste	0.47±0.05
Orange peel	0.58±0.07
Molasses	0.98±0.12
Wheat bran	0.56±0.12
Agriculture waste	0.32±0.08

The table indicates the average value of three experiments for cellulase activity with different raw materials. (Mean ± SD). When the results of molasses, sugarcance bagasse and paper pulp were compared with agriculture waste, there was significant different found in the enzyme activity where $p<0.05$ was obtained in one way ANOVA.

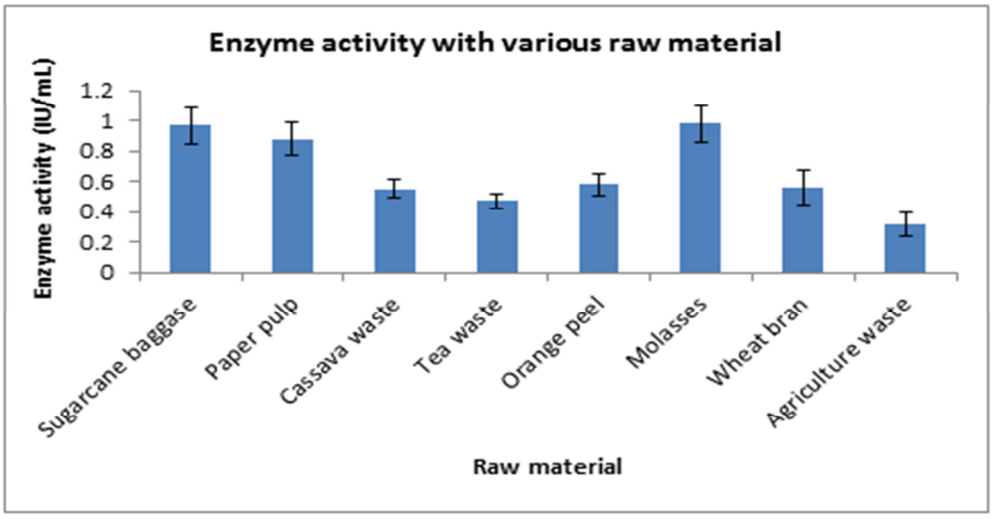

Fig 2. Activity of cellulase with different raw substrates

Studies have shown that sugarcane bagasse and molasses both contain sucrose and glucose along with cellulose molecules. These molecules enhance the growth rate of microorganisms leading to production of higher microbial populations which utilize cellulose faster by producing cellulase enzymes. [6,23] Studies have shown that fungi have more potential for production cellulase as compared to bacteria. *Aspergillus* species, *Penicillium* species, *Rhizomucor* species and *Trichoderma* species are among the leading fungi.[2,4,16,23] Solid state fermentation was found as the most efficient method for large scale production of enzyme using sugarcane baggase.[26] Many times pretreatment of cellulose is

carried to enhance the rate of production. This treatment includes chemicals like NaOH, H_2SO_4, H_2O_2. [27-29] In addition to chemicals, cellulose substrates are also subjected to destruction by autoclave. [29] Molasses was found to be one of the most economic sources for enzyme production. [22,30] Molasses is a by-product of the sugarcane industry and has vast applications especially in alcohol industries. [2,6] In case of agriculture waste and tea waste no free sugars are available to be utilized by microorganisms; hence it has to be completely dependent on the cellulose only for its growth. [31-34] Processing of agricultural waste by chemical and physical methods enhances the cellulase production rate. [32,33] Since cellulose degradation is a slow process it will take time hence the rate of production of cellulase with these sources are very less. Paper pulp contains partially digested cellulose which can be easily broken down by enzyme cellulase with better enzymatic activity. [35] Studies have shown that xylanase, cellulase and pectinase synergistically degrade long polymers and make it suitable for paper production. [36-38] However, excessive use of these enzymes should be avoided to maintain the structure stability. [38] Use of orange peel for production of cellulase is a low cost and environmental friendly approach. The limitation is availability of huge quantities of peels as waste material and its pretreatment with chemicals. [32,39,40] Here a moderate level of cellulase was obtained with orange peel in this study having a value of 0.58±0.07 IU/mL. In many studies wheat bran was also used as precursor for cellulase production, but they have observed that different composition of wheat bran affect significantly in production of cellulose. [3,23] Hence for each type of wheat bran method standardization may be required. Cassava waste contains 13% – 40% of cellulose which makes it less favourable for large scale production of enzyme. Lower concentration of cellulose in cassava waste may require more quantity of raw material and longer duration for production of enzyme. [26,41,42]

5. CONCLUSION

Based on the entire study, it was concluded that for production of extracellular cellulase by *B. amyloliquefaciens* sugarcane bagasse and molasses are best raw materials. Among both the sources, molasses could be a better source as it is easily available in large quantities. It was also concluded that waste material of cassava, agriculture and tea should be avoided as they give very less activity.

6. AUTHORS CONTRIBUTION STATEMENT

Ms. Nimisha Patel has carried out the entire experiment and analyzed the data. Dr. Ajit K Gangawane has planned the entire experiment and guided for execution. He has also helped in analysis of data.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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