

CELLULASE PRODUCTION BY *Bacillus subtilis* M1 USING PRETREATED GROUNDNUT SHELL BASED LIQUID STATE FERMENTATION

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ABSTRACT

Groundnut shell which is rich in natural cellulose was assessed as a substrate for production of cellulase enzyme by cellulolytic bacteria. In the present investigation the bacterial isolate *Bacillus subtilis* M1 was found to be capable of producing high amount of endoglucanase and exoglucanase on alkali treated groundnut shell. The effect of some nitrogen sources, amino acids and Ca⁺⁺ ions in the medium containing pretreated groundnut shell were also evaluated. It was observed that 2% substrate concentration, 1 mM calcium concentration were optimum for cellulase production. Ammonium nitrate was found to be the best among nitrogen sources tested. Asparagine, tryptophan and methionine were found to be stimulatory for cellulase activity.

Keywords: *Bacillus subtilis*, cellulase, endoglucanase, exoglucanase, groundnut shell

INTRODUCTION

Microbial cellulases consist of three types of hydrolytic enzymes: 1. endo-(1,4)- β -D-glucanase (also known as endocellulase, carboxymethyl cellulase and endoglucanase) which makes random cleavages at the β -glycosidic linkages of cellulose; 2. exo-(1,4)- β -D glucanase (also called cellobiohydrolases and exocellulase) which hydrolyses cellobiose units from the termini of cellulose chain; and 3. β -glucosidase (synonym cellobiase) which releases glucose from cellobiose and short chain oligosaccharides (Kim *et al.* 2008). The cellulases form of a very important group of enzymes that find applications in a variety of industries including textile industries, paper and pulp industries, food processing industries, wine and brewery industry etc. (Kuhad *et al.* 2011). However, the cost of enzymes poses a problem to their large scale utilization, especially for the environmentally crucial processes like production of biofuels (Brijwani *et al.* 2010). So, there is a need to search cheaper methods for producing

these enzymes. One of the possible methods for achieving this, is by using lignocellulosic wastes for the production of cellulases. Lignocellulosics offer a promising solution, since they are abundantly available and are produced in large amount as agricultural by-products.

Organic waste from renewable forest and agricultural residues are rich source of cellulose, hemicellulose and lignin (Brauns & Brauns 1960) and therefore, can be possibly used in fermentation process for the production of cellulases. Groundnut (*Arachis hypogaea* L) is an important oil seed crop of India. The pod or dry pericarp contains about 25-40% shell (Dey *et al.* 2002). The compositional analysis of groundnut shell indicates that the shell contains cellulose (65.7%), carbohydrates (21.2%), protein (7.3%), minerals (4.5%) and lipids (1.2%) (Masenda 2004). Groundnut shell is used as manure (Rao *et al.* 2009), used in mushroom cultivation (Jain & Vyas 2002), and in production of extracellular enzymes (Vyas *et al.* 2005).

The capacity of selected *Bacillus subtilis* to produce and secrete large quantities of

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extracellular enzymes has placed them among the most important industrial enzymes producers (Schallmey *et al.* 2004). This study reports *Bacillus subtilis* M1 as potent producer of cellulase enzymes on pretreated groundnut shell.

MATERIALS AND METHODS

Organism

Bacillus subtilis M1 was isolated from decomposing organic waste containing cellulose. Bacteria was isolated by enrichment method (Hans & Srinivasan 1968) for which 1 g mixture of rotting cellulosic substrates was inoculated into the isolation medium consisting of mineral salts solution supplemented with 0.1% yeast extract and strip of filter paper. After 3 to 7 days of incubation at 30 °C on reciprocal shaker, a patch of a yellow pigmented material appeared at the liquid air interface on the filter paper. As soon as the pigmented material appeared, a portion of filter paper was transferred with sterile wire and inoculated into fresh medium. The process was repeated several times to enrich aerobic and mesophilic cellulose utilizing organisms. The filter paper from the enriched culture was removed, macerated in a small amount of sterile water and streaked on the plates containing carboxy methyl cellulose agar. The bacterial colony was purified and maintained on Nutrient Agar Medium slants at 4 °C.

Substrate

GS (groundnut shell) was collected from local suppliers in and around Sagar District, Madhya Pradesh, India. Shell (1 kg) was dipped in water (5 L) to remove any amount of soluble sugar present in the substrate and then dried at 80 °C for 36 hours in electric oven and then chopped into small pieces (Krishna 1999), which were then ground in an electric grinder and sieved through 100 µm mesh sieve and kept at room temperature.

Pretreatment of GS

Powdered groundnut shell was treated separately with alkali (NaOH) and designated as sample ATRT (alkali treated), while acid (HCl) treated shells were labeled as sample AcTRT (acid treated). Dried and powdered shell (100 g) was

treated for 24 hours with 0.25N NaOH (500 mL) (Kojam *et al.* 2000) and with 0.25N HCl (500 mL). After treatment the powdered shell was repeatedly washed with distilled water until it reached neutral pH 7 and then dried over night at 60 °C.

Inoculum Preparation

Bacterial inoculum consisted of 2 mL of 24 hours old culture grown on nutrient broth giving absorbance of 1.2 at 660 nm.

Fermentation Medium (Submerged)

Mandels and Reese medium (Mandels & Reese 1957) was used for cellulase production. Chemical composition of medium was Proteose peptone, 1.0; (NH₄)₂SO₄, 1.4; KH₂PO₄, 2.0; NH₂-CO-NH₂, 0.3; MgSO₄·7H₂O, 0.3; CaCl₂, 0.3; FeSO₄·7H₂O, 0.005; MnSO₄·H₂O, 0.0016; ZnCl₂, 0.0017; and pretreated lignocellulosic substrate (GS), 10 g/L; 1,000 mL distilled water; pH 5.3.

Cellulase Production

The medium (25 mL) was dispensed in 150 mL conical flask and autoclaved at 15 psi for 15 minutes. The flasks were inoculated with bacterial culture and incubated at 37±1 °C under stationary conditions. The samples (10 mL) were withdrawn aseptically after the 6th day of incubation and supernatant was obtained after centrifugation at 9,500 rpm for 20 minutes. The supernatant obtained was used for enzymatic activity. The control consisting of untreated GS was also run simultaneously. All tests were performed in duplicate.

Enzyme Assay

Endoglucanase and exoglucanase activities were measured (Mandels 1974) in terms of International Unit (IU), which is micromoles (µm) of glucose released/minute/mL.

RESULTS AND DISCUSSION

Cellulases are a group of industrially important enzymes and their demand is increasing day by day with the growth of enzyme industry. Groundnut shell was assessed as cheap alternative lignocellulosic substrate for producing

cellulase enzymes since it is known to possess high percentage of cellulose (Masenda 2004). However, the lignin present in the groundnut shell can impede the cellulase enzymes. So, delignification was performed through alkali (NaOH) and acid (HCl) pretreatment. In the present investigation, the isolate M1 was found to be potent producer of cellulase enzymes on pretreated groundnut shell. It was isolated from decomposing organic waste containing cellulose as per the enrichment method described by Hans and Srinivasan (1968). Microorganism found was gram positive, spore producing, catalase positive rod shaped bacterium. It was identified as *Bacillus subtilis* by IMTECH (Institute of Microbial Technology), Chandigarh, India.

Under the conditions of pH 5, temperature 50 °C and an incubation period of 6 days, the

bacterial isolate *B. subtilis* M1's endoglucanase activity was found to be 2 fold (0.314 IU/mL) and exoglucanase activity was found to be 3.3 fold (0.043 FPU/mL) in ATRT GS as compared to untreated GS. In AcTRT GS, 1.5 fold (0.219 IU/mL) increased endoglucanase and 1.5 fold (0.020 FPU/mL) exoglucanase activity was obtained as compared with the untreated. So, the pretreatment resulted in higher enzyme production and the alkali treatment was found to be more efficient than the acid treatment (Fig 1). Alkali (NaOH) treatment of GS was found to be more superior to acid (HCl) treatment as it modifies lignocellulosic substrate by increasing the pore size and solubilizing lignin and hemicellulose. It increases the surface area of cellulose, reducing its crystallinity (Ming *et al.* 2008).

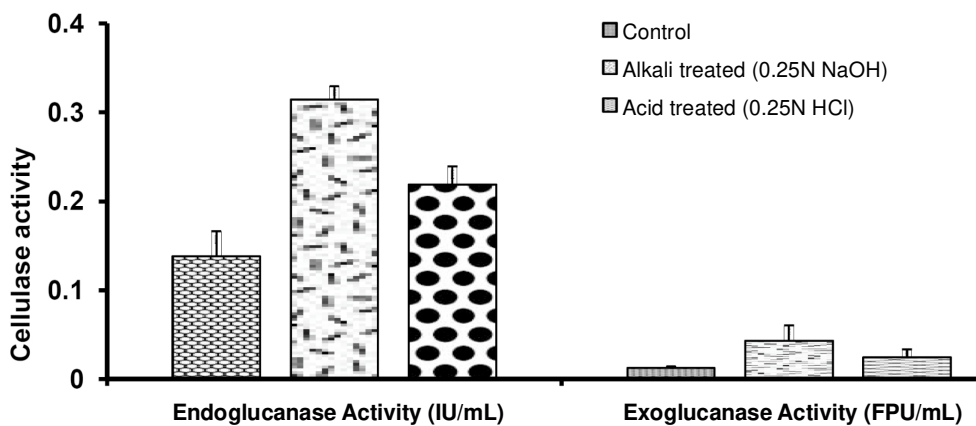


Figure 1 Effect of alkali and acid treatments of groundnut shell on production of cellulase by *Bacillus subtilis* M1

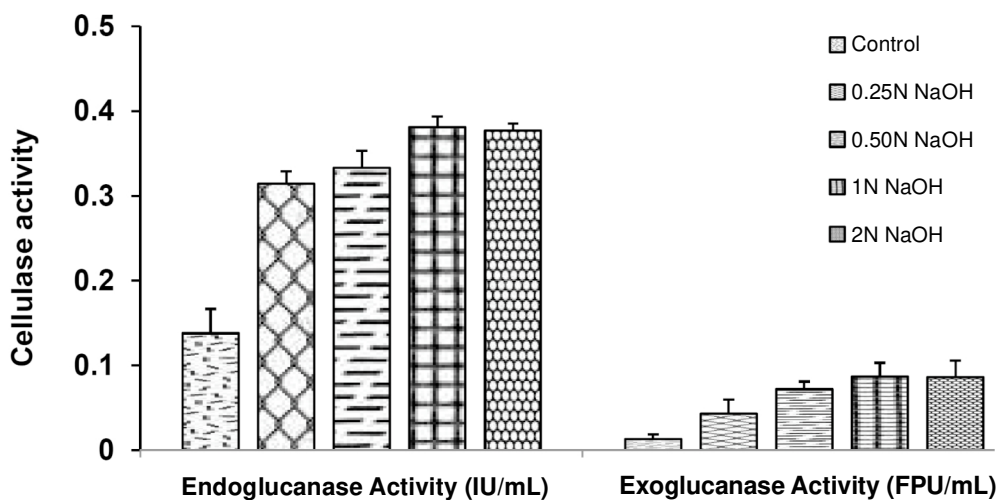


Figure 2 Effect of alkali pretreatment of different concentrations on groundnut shell on cellulase production by *Bacillus subtilis* M1

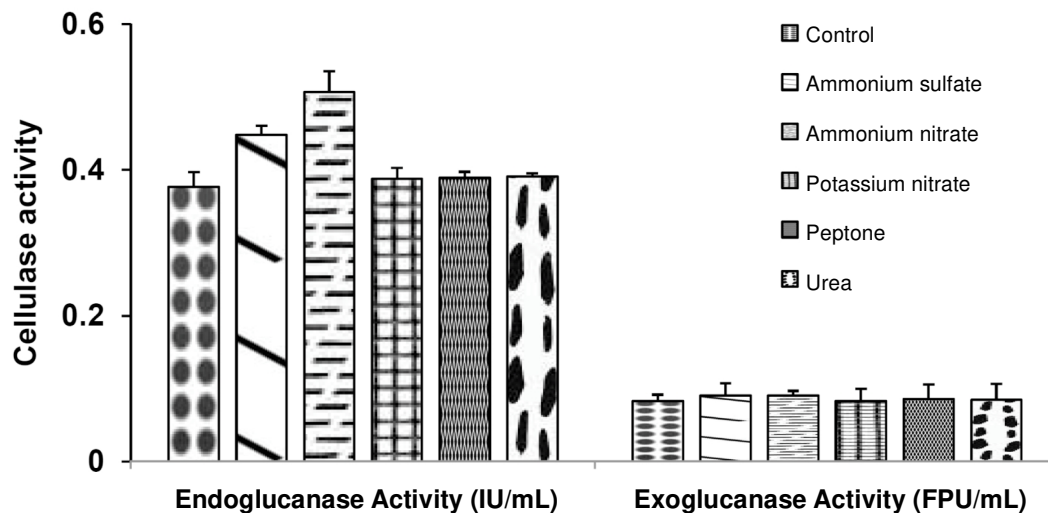


Figure 3 Effect of nitrogen sources on cellulase production on alkali treated groundnut shell by *Bacillus subtilis* M1

Pretreatment of powdered GS at different alkali concentrations (0.25N, 0.5N, 1N, 2N NaOH) showed that *B. subtilis* M1 produced the highest endoglucanase (0.381 IU/mL) and exoglucanase (0.087 FPU/mL) activities in case of 1N NaOH treated GS. Endoglucanase activity was increased by 2.7 fold and exoglucanase activity by 6.9 fold higher compared to untreated GS (Fig 2). The increased cellulase activity at 1N NaOH concentration might be due to increased solubilization of lignin and increase in swelling of cellulose I to cellulose II. Aguiar (2001) also reported 1N NaOH pretreatment of lignocellulosic substrates as best source for cellulase production. Chemical pretreatment of lignocellulosic material followed by its hydrolysis suggested that accessible surface area is a key determinant for enhancing cellulase yield. However, Sarkar and Aikat (2012) reported the highest activity of cellulases by *Aspergillus fumigatus* NITDGPKA3 on rice straw pretreated with 0.5MNaOH.

The amount of nutrients present in the production medium is one of the major factors impacting the microbial enzyme production. The effect of five nitrogen sources (ammonium sulfate, ammonium nitrate, potassium nitrate, peptone, urea) were tested separately (Fig 3).

Each nitrogen source was added in equivalent amount to the total nitrogen present in the medium (587 mg nitrogen/L medium) keeping the available nitrogen constant. Ammonium nitrate was found to be the best for endoglucanase

activity (0.507 IU/mL) and exoglucanase activity (0.091 FPU/mL) followed by ammonium sulfate, potassium nitrate, peptone and urea. Nitrogen is the major constituent of protoplasm and building block of enzymes (proteins). Sethi *et al.* (2013) also reported ammonium salt to be excellent source of nitrogen for cellulase production and concluded that this may be due to their direct entry of ammonium ion in protein synthesis.

Similarly, amino acids are nitrogen containing organic compound and therefore, have influence on cellulase synthesis, but the specific side groups present on the amino acids can also play some role in cellulase synthesis. Among various amino acids added to the medium (0.2 w/v), Tryptophan (0.675 IU/mL), asparagine (0.629 IU/mL) and methionine (0.564 IU/mL) were found to be the better suited for endoglucanase activity (Fig 4). On the other hand, alanine, arginine and threonine were found to be suppressive in action. *B. subtilis* M1 showed maximum exoglucanase activity in the presence of asparagine (0.126 FPU/mL) followed by methionine (0.108 FPU/mL) and tryptophan (0.095 FPU/mL). Amino acids, being the building blocks of protein, have a profound influence in the cellulase synthesis of bacteria. The increased production might be due to the enhanced synthesis of cellulolytic enzymes in the presence of amino acids. Asparagine and methionine were found to be stimulatory for cellulase activity in *Aspergillus terreus* AV49 by Vyas *et al.* (2005).

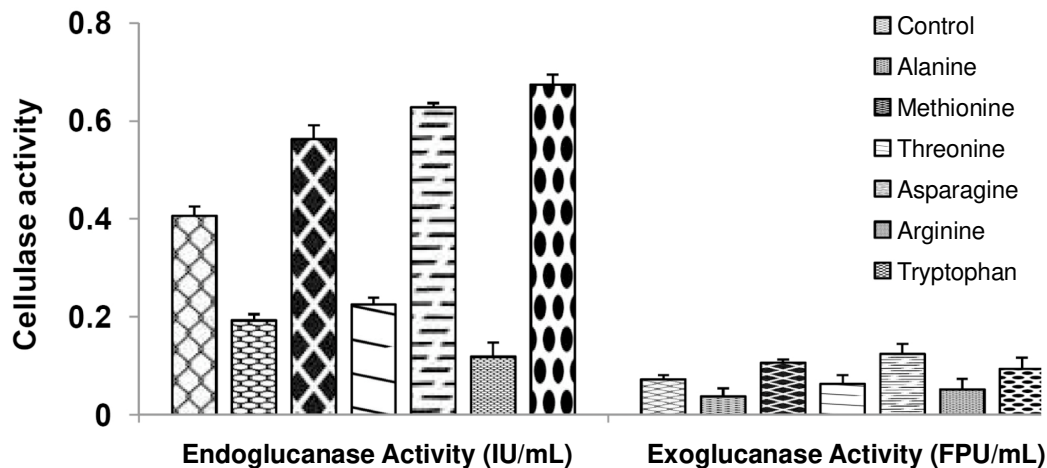


Figure 4 Effect of different amino acids on cellulase production on alkali treated groundnut shell by *Bacillus subtilis* M1

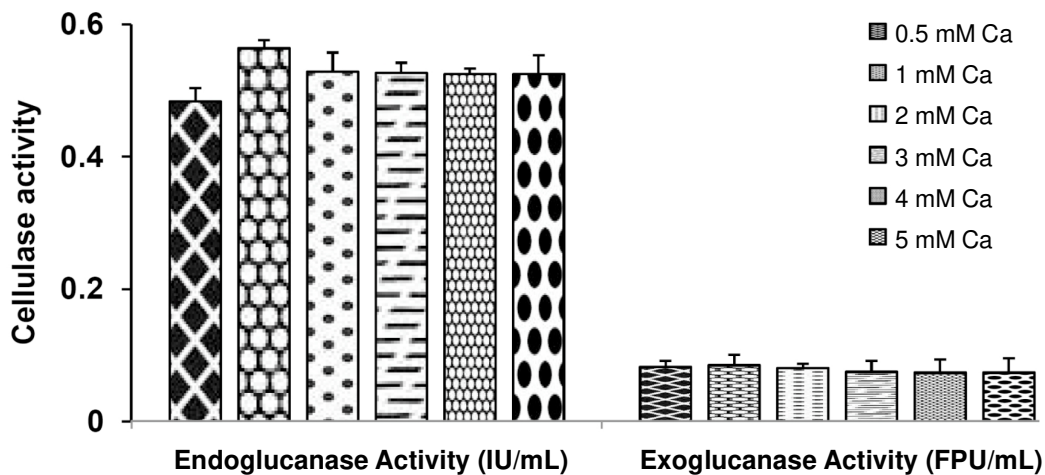


Figure 5 Effect of calcium concentration on cellulase production on alkali treated groundnut shell by *Bacillus subtilis* M1

The activity and stability of various enzymes are known to be impacted by various ions. Different concentrations of calcium (0.5-5 mM) had differential impact on the enzymatic activity (Fig 5). Low (1 mM) concentration produced maximum endoglucanase activity (0.564 IU/mL). However, at higher concentration (2-5 mM) endoglucanase activity was almost static, yet remained higher than control. Exoglucanase activity was maximum (0.085 FPU/mL) at 1 mM concentration and thereafter, it decreased up to 5 mM.

Metal ions are generally cellulase stimulators at low concentration, whereas inhibitory at very high concentration. Ca^{++} was found to positively influence the activity of cellulase at concentrations less than 1 mmol/L by Wang *et al.*

(2012). In the present study, Ca^{++} (1 mM) was found to be stimulatory and higher concentration showed static endo- and exoglucanase activities by *B. subtilis*. Ca^{++} (10 mM) is reported (Kim *et al.* 2001) to be stimulatory for cellulase (exoglucanase) activity in *Trichoderma reesei*. Strong affinity and tightness of the enzymes would disrupt the hydrogen bonding network in the crystal lattice, ultimately collapsing the ordered structure of cellulose particles and preventing adhesion. Kotchoni *et al.* (2003), however, reported enhanced cellulase activity at low calcium concentration (1 mM) in *Bacillus pumilus* BpCRI6.

The effect of substrate concentration is also known to impact the enzyme activity. In the present investigation it was observed that with

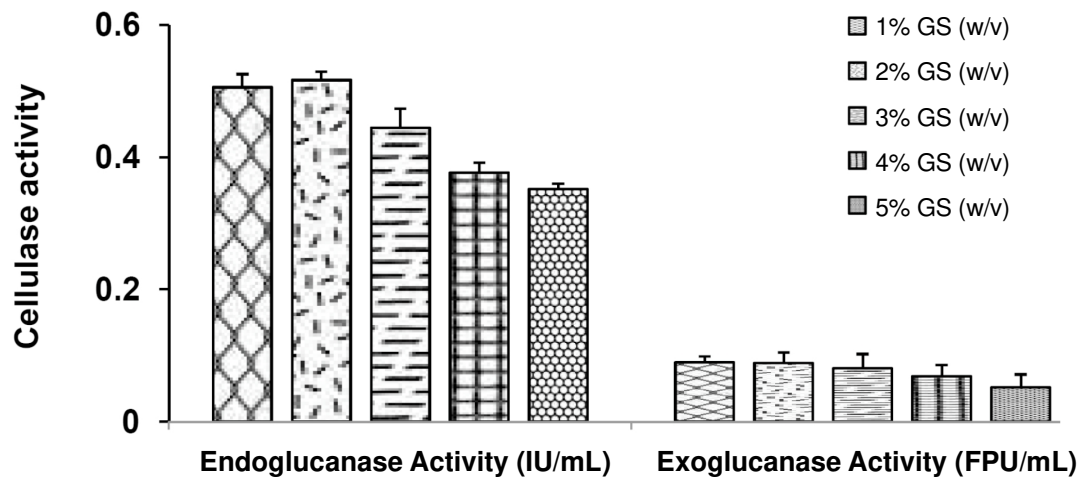


Figure 6 Effect of substrate concentration on cellulase production on alkali treated groundnut shell by *Bacillus subtilis* M1

increased substrate concentration, the endoglucanase activity showed increasing trend up to 2% substrate concentration (0.517 IU/mL), thereafter, no further increase in activity was observed (Fig. 6). Maximum exoglucanase activity was observed at 1% substrate concentration (0.090 FPU/mL). Okonkwo (2014) also observed maximum activity at 1% substrate concentration in case of cellulase produced by *Aspergillus flavus*.

CONCLUSIONS

The present findings raise the possibility of using groundnut shell as a substrate for production of cellulase enzymes by *Bacillus subtilis* M1. The cultural conditions as well as the type of pretreatment also seem to have profound impact on the enzyme activity. There is a need to assess the production at pilot plant scale, so that it can be used subsequently at the commercial scale in the long run.

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