Strain Improvement of *Bacillus coagulans* and *Geobacillus stearothermophilus* for Enhanced Thermostable Cellulase Production and the Effect of Different Metal Ions on Cellulase Activity

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Abstract

The current study was focused on the strain improvement of *Bacillus coagulans* and *Geobacillus stearothermophilus* for thermostable cellulase production with higher enzyme activity. For strain improvement UV radiations, NTG and Sodium azide were used as mutagenic agents.NTG was found to be best mutagenic agent among all in term of producing the mutants with highest cellulase activity. Mutant strain C11 exhibited the highest cellulase specific activity at 45 U/mg followed by C15 (39 U/mg) in case of *B. coagulans* while Mutant strain S18 exhibited the highest cellulase specific activity at 69 U/mg followed by S12 (62 U/mg) in case of *G. stearothermophilus*. Specific activity of cellulase was highest in both the cases on addition of Co^{2+}, 92 U/mg in case of *B. coagulans* C11 and 118 U/mg in case of *G. stearothermophilus* S18. Ag^{+}, Mg^{2+}, Se^{2+},Ca^{2+},Co^{2+},Mn^{2+},K^{+}, Zn^{2+} ,Fe^{3+}, Hg^{2+} and Cu^{2+} showed positive change in specific activity while Na^{+}, Ni^{2+} negative change in specific activity of cellulase with respect to specific activity of cellulase in absence of any additive in case of *B. coagulans* C11 and Ag^{+}, Mg^{2+}, Se^{2+},Co^{2+},Mn^{2+} and Hg^{2+} showed positive change in specific activity, Na^{+}, K^{+} showed no change in specific activity while Ca^{2+}, Zn^{2+}, Ni^{2+}, Fe^{3+} and Cu^{2+} showed negative change in specific activity of cellulase with respect to specific activity of cellulase in absence of any additive in case of *G. stearothermophilus* S18.

Keywords: Assay, Cellulose, Enzyme Activity, Mutants, Metal Ions.

INTRODUCTION

Cellulose is considered to be the primary product of photosynthesis and the most abundant renewable carbon resource in nature. Cellulose is the homo polymer of a β (1→4) linked glucose molecules. In the present time when petroleum fuel resources are decreasing day by day because of high consumption, cellulosic biofuels are the future alternative of present non renewable petroleum fuels but the production cost of cellulase is still the major cost factor in the hydrolysis of cellulosic materials to fermentable sugars. It is therefore essential to improve the production of cellulase in order to make the process more economically viable and efficient. The cellulase complex basically consists of three major components, endocellulase (EC 3.2.1.4), exocellulase (EC 3.2.1.91) and cellobiase (EC 3.2.1.21). Endocellulase cleaves internal bonds at amorphous sites that create new chain ends; exocellulase cleaves two to four units from the ends of the exposed chains which are produced by endocellulase while cellobiase hydrolyses the exocellulase product into individual monosaccharides. There are many factors like feedback inhibition and repression, which limit the production and activity of cellulase enzyme. To overcome these problems, best way is to improve and optimize the microbial strains for cellulase production by mutation. There are many strategies of mutagenesis like ultraviolet (UV), X-rays, gamma radiation, ethyl methane sulfonate (EMS), N-methyl-N´-nitro-N-nitrosoguanidine (NTG) and atmospheric pressure non equilibrium discharge plasma (APNEDP). *Streptomyces pseudogriseolus*, UV mutant, induced over-production of xylanase (161% production improved) as compared to the wild type (Abdel-Aziz *et al.*, 2011). Sequential treatment by γ-irradiation, UV and NTG was also utilized for strain improvement in *Aspergillus sp.* The activities of carboxymethyl cellulase, filter paper cellulase and β-glucosidase of cellulase by *Aspergillus sp.* XTG-4 were improved by 2.03 fold, 3.20 fold and 1.80 fold, respectively when compared to wild strain (Vu *et al*., 2009). The current study was focused on the strain improvement of *Bacillus coagulans* and *Geobacillus stearothermophilus* for thermostable cellulase production as both these bacteria are thermophilic in nature (Odeniyi *et al.*, 2009 and Makky *et al.*, 2009).

MATERIALS AND METHODS

A. Microbial Culture and Growth Media

Wild type *Bacillus coagulans* and *Geobacillus stearothermophilus* were isolated from rice field soil and were identified by 16 S rRNA sequencing technique (Woo *et al*., 2008). Carboxymethylcellulose was taken as the growth media for cellulolytic bacteria. Other nutrients include - Agar = 11 g, CMC = 20 g, $NH_4H_4PO_4$ = 0.5 g, KCl = 0.1 g, $MgSO_4$.$7H_2O$ = 0.5 g, Yeast Extract = 0.5 g (Each Constituent is per 1000 ml)

B. Strain Improvement

a. Mutagenesis by Ultravoilet (UV) Irradiation

Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than X-rays, that is, in the range 10 nm to 400 nm, corresponding to photon energies from 3 eV to 124 eV. Bacterial cells were spread on CMC medium plates. The plates were placed under a UV lamp at a distance of 40 cm and were irradiated for various periods of time. Following irradiation, the plates were kept in the dark for 2 hours and then were spread onto CMC plates and incubated at 45°C for 48 hours, the number of colonies was counted to determine survival rates after exposure to UV radiations. Afterward, plates were stained by Congo red.

b. Mutagenesis by N-methyl-N´-nitro-N-nitrosoguanidine (NTG)

N-methyl-N´-nitro-N-nitrosoguanidine (NTG) is a potential mutagenic agent with high capability of inducing the mutations. The wide type cells grown in CMC medium at 45°C for 24 hour was harvested at logarithmic phase by centrifugation at 14000 rpm for 10 minutes at 4°C and washed twice with citrate/phosphate buffer (containing 0.1 M citric acid and 0.2 M phosphate buffer) pH 5.0. NTG, 0.1g/ml, was added into the cell suspension. After incubation for 2 hour at 45°C with rotation speed at 100 rpm in shaker incubator, the cells were centrifuged and washed immediately with buffer. The treated sample was transferred into CMC plates. The plates were stained by Congo red to see the cellulolytic activity after incubated at 45°C for 48 hours.

c. Mutagenesis by Sodium azide (NaN_3)

Sodium azide is acutely toxic and highly mutagenic. The wide type cell grown in CMC medium at 45°C for 24 hour was harvested by centrifugation at 14000 rpm for 10 minutes at 4°C and washed twice with citrate/phosphate buffer, pH 5.0. Sodium azide, 0.1mg/ml, was added into the cell suspension. After incubation for 2 hour at 45°C with rotation speed at 100 rpm in shaker incubator, the cells were centrifuged and washed immediately with buffer. The treated sample was transferred into CMC plates. The plates were stained by Congo red to see the cellulolytic activity after incubated at 45°C for 48 hours.

C. Screening and Selection for the Highest Cellulase-Producing Mutants

The mutants were selected from CMC plates after mutagenesis on the basis of clearance zones appearing in detection media by using Congo red. The mutants, showing higher cellulase activity were again mutated by same method described above and this procedure was repeated four times to get the mutants with maximum cellulase activity. The improved mutated strains were then subcultured on CMC plates further to get pure colonies of these mutants and these mutants were further used to observe the effects of metal ions on cellulase production.

D. Effects of Metal Ions on Cellulase Specific Activity

Different metal ions were used as additives to check their effect on cellulase specific activity. These included Ag^{+},Mg^{2+},Na^{+},Se^{2+},Ca^{2+},Co^{2+},Mn^{2+},K^{+},Zn^{2+},Ni^{2+},Fe^{3+}, Hg^{2+} and Cu^{2+}.All of them were used in concentration of 1mM. The crude enzyme was incubated separately with the additives in citrate buffer (0.05 M, pH 6.0) at 45°C for 1 hour prior to measuring the cellulase activity.

E. Cellulase Assay

Cellulase assay was done by Dinitrosalicylic Acid (DNS) method (Ghose, 1987) by using a reaction mixture containing 1 ml of crude enzyme solution with 1 ml of 1% CMC (incubated at 40°C for 30 min) in Citrate/Phosphate buffer (pH 5).

F. Crude Enzyme Extraction

Bacteria were grown in CMC-Nutrient media for 3 days,then broth was centrifuged at 14000 rpm for 20 minutes, supernatant was taken as crude enzyme extract. This crude enzyme extract was stored in Citrate/Phosphate Buffer at pH 5 at 4°C.

G. Protein Assay

Total Protein estimation was done by Lowry's Method (Oliver H. Lowry, 1951)

RESULTS

Strain Improvement by UV, NTG and Sodium azide Mutagenesis

The wild type *B. coagulans* and *G. stearothermophilus* were subjected to successive mutagenic treatment using UV irradiation, Sodium azide and NTG. After strain improvement, 112,138 and 169 mutant colonies were obtained from UV, Sodium azide and NTG treatment in case of *B. coagulans* and 194,216 and 223 mutant colonies were obtained from UV, Sodium azide and NTG treatment in case of *G. stearothermophilus*. Based on the ratio of diameter between the clearing zone and colony on the CMC-Congo red medium (Sangkharak *et al*., 2012. The cellulase activity of clones that displayed the largest clearing zones was assessed after 48 hours of cultivation. The twenty best isolates (strain C1 to C20 in case of *B. coagulans* and S1 to S20 in case of *G. stearothermophilus*) were selected and cultivated in 50 ml of CMC medium for enzyme production. Mutant strain C11 (NTG Mutant) exhibited the highest cellulase specific activity at 45 U/mg followed by C15 (UV Mutant) (39 U/mg) in case of *B.coagulans* while Mutant strain S18 (NTG Mutant) exhibited the highest cellulase specific activity at 69 U/mg followed by S12 (Sodium azide Mutant) (62 U/mg) in case of *G. stearothermophilus*. The least cellulase specific activity was seen in case of C02 in case of *Bacillus coagulans* mutants and it was 3 U/mg while in case of *Geobacillus stearothermophilus* mutants, it was seen in S14 and it was 7 U/mg. The assayed enzyme activity indicated the differences among the mutants of *B.coagulans* and *G. stearothermophilus* and their respective wild types. The Cellulase Specific activity was 2.091 U/mg for *B. coagulans* wild strain and 3.47 U/mg for *G. stearothermophilus* wild strain. Under optimal condition, the cellulase activity achieved from *Cellulomonas sp.* TSU-03 strain M23 was found to be 1.30 fold higher than that of wild type (Sangkharak *et al.,* 2012).The results concluded that mutagenesis by UV and NTG caused changes in cellulase production or secretion. Therefore, strain C11 and M18 were selected and used throughout this study.

Table 1. Mutant Strains and their respective Cellulase Specific Activity

B. coagulans **Mutant Strain**	**Cellulase Specific Activity(U/mg)**	***G. stearothermophilus*** **Mutant Strain**	**Cellulase Specific Activity (U/mg)**
Wild Strain	2.091	Wild Strain	3.47
C01	31	S01	42
C02	3	S02	36
C03	27	S03	14
C04	8	S04	22
C05	11	S05	35
C06	15	S06	51
C07	26	S07	11
C08	12	S08	29
C09	19	S09	42
C10	4	S10	26
C11	**45**	S11	39
C12	22	S12	62
C13	6	S13	17
C14	29	S14	7
C15	39	S15	23
C16	5	S16	33
C17	20	S17	13
C18	32	**S18**	**69**
C19	14	S19	18
C20	34	S20	55

Effects of Metal Ions on Cellulase Specific Activity

Co^{2+} is having maximum positive effect on cellulase specific activity in case of both the bacterial mutant strains, specific activity of cellulase was 92 U/mg in case of *B.coagulans* C11 and 118 U/mg in case of *G. stearothermophilus* S18. Ag^{+}, Mg^{2+}, Se^{2+},Ca^{2+},$Co^{2+,}Mn^{2+}$,K^{+}, Zn^{2+} ,Fe^{3+}, Hg^{2+} and Cu^{2+} showed positive change in specific activity while Na^{+}, Ni^{2+} negative change in specific activity of cellulase with respect to specific activity of cellulase in absence of any additive in case of *B.coagulans* C11 and Ag^{+}, Mg^{2+}, Se^{2+},$Co^{2+,}Mn^{2+}$ and Hg^{2+} showed positive change in specific activity, Na^{+}, K^{+} showed no change in specific activity while Ca^{2+}, Zn^{2+}, Ni^{2+}, Fe^{3+} and Cu^{2+} showed negative change in specific activity of cellulase with respect to specific activity of cellulase in absence of any additive in case of *G. stearothermophilus* S18. As per Mawadza *et al*., 2012 ,K^{+}, Na^{+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+} and Fe^{3+} did not influence the activity of the enzymes while Co^{2+}, Ag^{+}, Mn^{2+} and Hg^{2+} showed increase in cellulase activity of Bacillus strains.

Table 2. Metal Ions as additives and their effect on Cellulase Specific Activity

Additive (1mM)	**Specific Activity of Cellulase (*B.coagulans* C11)**	**Specific Activity of Cellulase (*G. stearothermophilus* S18)**
Nil	45	69
Ag^{+}	+	+
Mg^{2+}	+	+
Na^{+}	-	No Change
Se^{2+}	+	+
Ca^{2+}	+	-
Co^{2+}	**+**	**+**
Mn^{2+}	+	+
K^{+}	+	No Change
Zn^{2+}	+	-
Ni^{2+}	-	-
Fe^{3+}	+	-
Hg^{2+}	+	+
Cu^{2+}	+	-

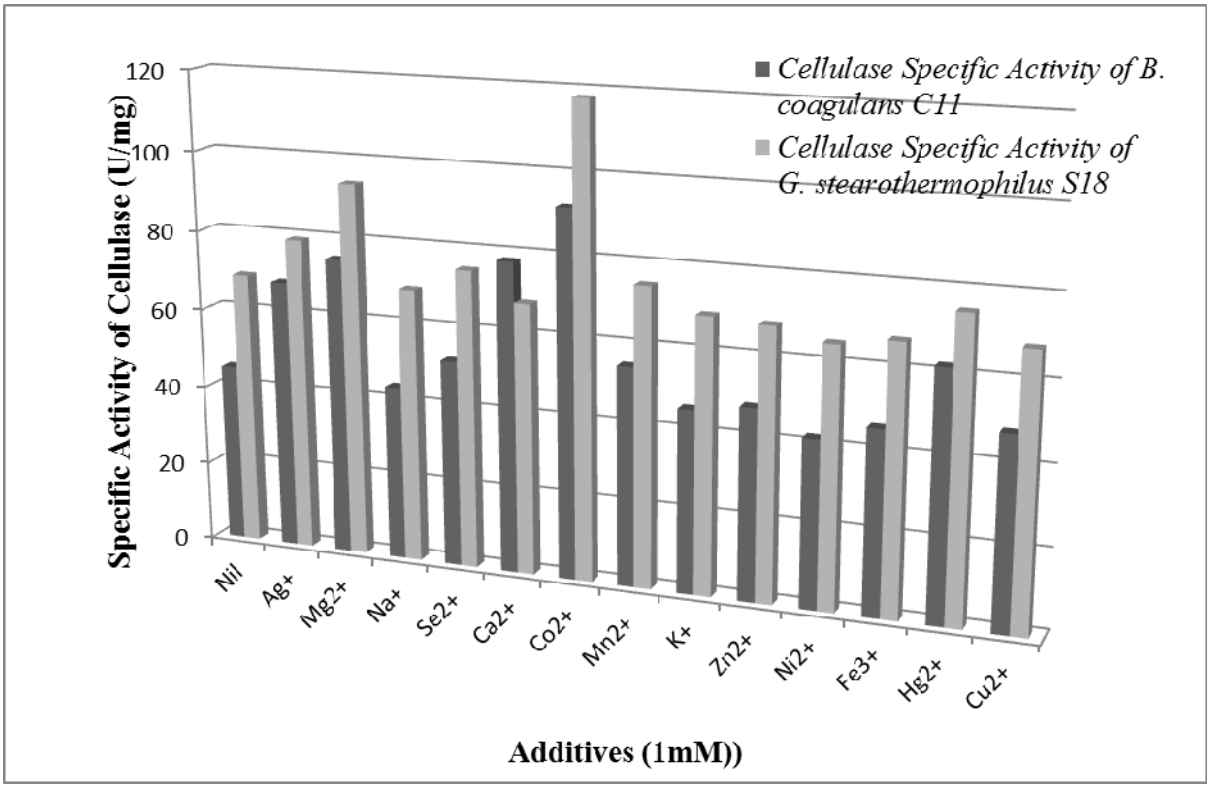

Fig. 1 Graph showing the effect of various additives on Cellulase Specific Activity.

CONCLUSION

The improvement of cellulase production from *B.coagulans* and *G. stearothermophilus* was attempted by the mutagenesis and optimization study. *B.coagulans* C11, a prominent strain, producing a high level of cellulase was selected from 112,138 and 169 mutant colonies which were obtained from UV, Sodium azide and NTG treatment in case of *B. coagulans* and *G. stearothermophilus* S18 was selected from 194,216 and 223 mutant colonies which were obtained from UV, Sodium azide and NTG treatment in case of *G. stearothermophilus*. Mutant strain C11 exhibited the highest cellulase activity at 45 U/mg followed by C15 (39 U/mg) in case of *B. coagulans* while Mutant strain S18 exhibited the highest cellulase activity at 69 U/mg followed by S12 (62 U/mg) in case of *G. stearothermophilus*. In both bacteria, NTG was found to be best mutagenic agent among NTG, UV and Sodium azide in respect of highest cellulase activity as both mutants C11 and S18 were NTG mutants. Further metal ions effect was observed on cellulase specific activity, which showed that Co^{2+} has the maximum increasing effect in both mutant strains *B. coagulans* C11 and *G. stearothermophilus* S18. The results suggested that *B. coagulans* C11 and *G. stearothermophilus* S18 had a good potential for production of cellulase by fermentation using optimized media with appropriate concentration of Co^{2+}.

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