

Wild type *Bacillus subtilis* treated with ethyl methyl sulphonate produced catabolite insensitive mutants with improved cellulase production

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Original article

Keywords: Cellulases, mutagenesis, carboxymethylcellulose, catabolite repression, glucose

Posted Date: March 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-278081/v1>

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Abstract

The goal of this present investigation was to mutagenize *Bacillus subtilis* with Ethyl Methyl Sulphonate (EMS), screen the mutants for cellulase production and evaluate the influence of different glucose concentrations on their cellulase production potentials. The wild type *B. subtilis* was treated with 20, 40, 60 and 80 µl of EMS and the mutants generated were screened for cellulase production in minimal salt medium containing carboxymethylcellulose (CMC) as the carbon source. Quantitatively, cellulase activity and protein contents were determined by dinitrosalicylic acid and Lowry methods respectively. Seven mutants were developed from each of the EMS concentration bringing the total to twenty-eight from all the concentrations. Approximately 14 and 57% of the mutants developed from 40 and 60µl of EMS had higher cellulase activities than the wild type, while none of the mutants developed from 20 and 80 µl of EMS had better activities than the wild type. The supplementation of 0.2, 0.5, 1.0 and 1.5% glucose in enzyme production medium caused approximately 100, 14, 29 and 14% cellulase repression respectively in the mutants developed from 60µl EMS. Mutants MSSS02 and MSSS05 were considered as catabolite insensitive mutants because their cellulase production were enhanced in comparison to wild type.

Introduction

In the last few decades of studying bioconversion of cellulose from lignocellulosic biomass, it has been affirmed that it is pivotal to the production of fermentation sugars, a template molecule for the synthesis of Adenosine Triphosphate (ATP), a renewable energy source in the biosphere (Romeo, 2008; Islam and Roy, 2018). Cellulose is a homopolymer of repeated units of D-glucose linked by β -1,4 glycosidic bonds. It is one of the major structural components of plant biomass in nature and most abundant biomass generated via various agricultural practices (Islam and Roy, 2018). Usually, a complete degradation of cellulose into glucose units is achieved by synergistic interaction of three types of cellulases namely: β -1,4 endoglucanases, cellobiohydrolase and β -D glucosidase (Perez et al., 2002; Bras et al., 2011). β -1,4 endoglucanases targets and hydrolyze exposed glycosidic bonds within the cellulose polymer, cellobiohydrolase liberates cellobiose from the reducing and nonreducing ends, while β -D glucosidase cleaves cellobiose and cello-oligosaccharide into glucose (Bras et al., 2011; Shanmugapriya et al., 2012). It is therefore imperative to develop research strategies for an effective utilization of cellulose containing wastes as cheap carbon sources.

Recently, microbial cellulases have attracted much attention because of exponential increase in the application of these enzymes in detergent, textile, food, leather, paper and feed industries. It has also found application in fiber modification, biomass fermentation and pharmaceutical purposes (Cherry and Fidantsef, 2003; Shanmugapriya et al., 2012). Industrial application of cellulases requires proper identification of promising microbial candidates with good cellulolytic machinery that are stable at extreme conditions such as high temperature and pH (Abdelnasser and Ahmed, 2007). Numerous cellulose-degrading microorganisms have isolated and identified with many studies laid more emphasis on cellulases from fungal origin due to the fact that they produced it in abundant, easier to extract from production medium and it has been commercialized (Saha et al., 2006; Doi, 2008). However, researchers

are now considering cellulase-producing bacteria because their growth rate is faster than fungi, they express multienzyme complexes and are resistant to extreme environmental stress (Waeonukul et al., 2009; Deswal et al., 2011; Marjamaa et al., 2013). Thus, cellulolytic bacteria are classified as alkaliphilic or acidophilic and thermophilic or psychrophilic. They have documented to often produced stable enzymes under harsh conditions for cellulose bioconversion process. Bacterial genera belonging to *Cellulomonas*, *Clostridium*, *Ruminococcus*, *Cellulosimicrobium*, *Thermomonospora*, *Erwinia*, *Streptomyces*, *Bacteriodes*, *Fibrobacter*, *Microbispora*, *Acetovibrio*, *Paenibacillus* and many more have been reported to produce cellulases when cultured aerobically or anaerobically in enzyme production media (Lo et al., 2009; Wilson, 2011).

The increasing demand for cellulases in various industrial process necessitates extension in both qualitative and quantitative improvement in microbial strains producing the enzymes. Overproduction of enzymes requires strain improvement since the yield from wild strains are usually too low such that the industrial demand could not met. Strain improvement in many experimental trials is usually achieved by extensive application mutagens such as Ultra violet radiation, N-methyl- N'-nitro-N-nitroso guanidine (NTG), Ethyl Methyl Sulphonate (EMS), Ethidium bromide and nitrous acid resulting in mutation and selection of improved clones (Olaniyi et al., 2014; Sadhu et al., 2014). In many occasions, the effect of these mutagens may be very lethal but sometimes, exposed organisms may develop better adaptation to harsh environment with improved biocatalytic efficiency (Femi-Ola, 2008; Sadhu et al., 2014).

Catabolite repression which is a bottleneck to continuous production of relevant industrial enzymes informed the current study and research efforts are now targeting catabolite insensitive strains. The microbial secretion of extracellular enzymes is regulated by induction and repression mechanism. The rate of cellulose hydrolysis mediated by cellulase has been reported to be impeded by the products generated by hydrolysis and fermentation especially when the two processes are carried out simultaneously (Lynd et al., 2002; Olaniyi et al., 2014). The presence of high content of readily available metabolized carbohydrate source and/or end product of sugar fermentation account for the repression of enzyme in most organisms and increased cost of enzyme production (Kayikc and Nielsen, 2015; Kim et al., 2019). It is hypothesized that catabolite insensitive mutants would bypass cellulase repression and permit high cellulase yield via direct bacterial hydrolysis and fermentation.

Bacterial identity was authenticated using conventional methods. *Bacillus subtilis* was exposed to different concentrations of EMS and emerged mutants (clones) were screened for cellulase production. Afterwards, wild type and the mutants were cultured enzyme basal media supplemented with different glucose concentrations. Mutants with higher enzyme activity in the presence of repressing sugar were considered as catabolite insensitive mutants. The mutagenesis of wild type *B. subtilis* by EMS led to the development of improved catabolite insensitive mutants.

Materials And Methodology

Collection of isolates

Bacillus subtilis CWB isolated from cassava waste water and previously confirmed to have xylanolytic activity capability was obtained from culture bank of the Department of Microbiology, FUTA for this study. The identity of the isolate was authenticated by sub-culturing on fresh nutrient agar and subjected to some biochemical tests. The outcome of which was compared with Bergey's Manual of Determinative Bacteria.

Mutagenesis of *Bacillus subtilis*

The isolate was mutagenized following the method of Olaniyi et al. (2014). *Bacillus subtilis* cultivated in nutrient broth for 24 hours was cold centrifuged at 4 °C, 4000rpm for 15mins and the supernatant was discarded. The cell debris was severally washed with normal saline, varied concentrations of Ethyl Methyl Sulphonate (EMS) 20µl, 40µl, 60µl and 80µl was added respectively and incubated at room temperature for 20mins. The mutagenized cells were washed with normal saline and thereafter diluted with Minimal Salt Medium (1g CMC, 0.1g yeast extract, 0.2g NaNO₃, 0.05 KCl, 1.4g KH₂PO₄, 0.06g MgSO₄.7H₂O, 0.1g Peptone) in a ratio 2:10 and incubated for 24 hours. The diluted mutagenized cells were cultivated in nutrient agar and incubated for 24 hours at 37 °C for individual mutants to emerge. Each emerged colony on the plate was considered to be a mutant. Each mutant encoded with a name is inoculated into a 5ml nutrient broth.

Primary screening of mutagenized cells for cellulase production

50 µl of broth culture of the mutant and wild type was inoculated in 5ml MSM into respective test tubes, incubated at 37°C for 24 hours and then centrifuged at 4000rpm for 15mins. 50µl of serially diluted supernatant from the dilution factor 10⁵ was introduced into wells bored in a carboxyl-methyl-cellulose (CMC)-nutrient agar plate and incubated at 37°C for 18-24 hours. The plates were flooded with 1% Congo red, allowed to stand for 15 mins at room temperature and afterward counterstained with 0.1M NaCl. Clear zones that appeared around wells indicates CMC hydrolysis. Clear zone around wells was measured in centimeter (mm) (Olaniyi et al., 2014).

Secondary screening of mutagenized cells for cellulase production

The mutants and wild type were screened quantitatively for cellulase production. Fifty microliter (50µl) of mutants and wild type were inoculated into 3ml of sterilized cellulase production medium consisting of 0.02g peptone, 0.02g yeast extract, 0.02g NaNO₃, 0.025g K₂HPO₄, 0.005g MgSO₄, 0.005g CaCl₂, 0.001g FeSO₄.7H₂O and 1g (CMC) dissolved in 100ml of sterile distilled water and incubated at 37°C for 24 hours. The medium was cold centrifuged at 6000rpm to obtain supernatant (crude enzyme) (Femi-Ola, 2008).

Enzyme assay

Five hundred microliter (500µl) crude enzyme was pipetted in a test tube containing 500µl CMC prepared in 50 mM phosphate buffer (pH 6.7). The mixture in test tubes were incubated in a water bath at 45°C for

15mins and the reaction was terminated by adding 1ml of DNSA and then boiled at 100°C for 10 mins for colour development. One milliliter (1ml) of distilled water was added to the test tube to bring it up to 2ml and then read at 540 nm using spectrophotometer (Waeonukul et al., 2009).

Protein determination

The protein content in cellulase production medium was determined as described by Lowry et al. (1951) using Bovine Serum Albumin (BSA) as a standard.

Catabolite insensitive mutants

All the mutants and wild type of *B. subtilis* were subsequently cultured in the enzyme production media supplemented with different concentrations of glucose (0.2g, 0.5g 1.0g and 1.5g w/v glucose) serving as energy source. Enzyme production and assay were performed as previously mentioned. Mutants that exhibited appreciable enzyme activity were considered as catabolite insensitive mutants (Olaniyi et al., 2014; Kim et al., 2019).

Results

Table 1 reveals the biochemical characterization of the isolate leading to its authentication. The isolate was positive to catalase, Gram's reaction, nitrate reduction, triple sugar iron, citrate, gelatin hydrolysis and negative to indole and oxidase. The isolate was tentatively identified as *B. subtilis*.

Table 2 shows the qualitative screening of cells mutagenized with EMS and wild type *B. subtilis* for cellulase production on CMC-agar medium. The mutants showed different hydrolysis zones on CMC-agar media indicating varied cellulase activities, the zone ranged from 2.8 to 6.5 mm. The highest zone of hydrolysis of 6.5 mm was obtained from the mutant designated M01, while mutant M08 showed the least zone of 2.8 mm. Approximately 57% of the mutants (MS04, MS03, MS02 and MS01) had higher zone of hydrolysis than the value obtained from the wild type. The mutants emerged from 40µl EMS treatment and wild type exhibited varied zones of hydrolysis on CMC-agar media. Mutant with code MSS03 was the only

Table 1
Morphological and biochemical identification of *Bacillus subtilis*

Biochemical Tests	Results
Cell shape	Rods
Gram reaction	Positive
Motility	Motile
Catalase	Positive
Indole	Negative
Triple sugar iron	Positive
Citrate	Positive
Gelatin hydrolysis	Positive
Oxidase	Negative
Nitrate reduction	Positive

Table 2
Hydrolysis zones on CMC-agar media by mutants of wild type *B. subtilis* developed from different concentrations of EMS

Mutant code	20 µl	Mutant code	40 µl	Mutant code	60 µl	Mutant code	80 µl
MS01	4.2	MSS01	3.0	MSSS01	3.6	MSST01	3.6
MS02	4.0	MSS02	3.1	MSSS02	3.9	MSST02	3.8
MS03	3.8	MSS03	5.1	MSSS03	5.5	MSST03	3.4
MS04	5.8	MSS04	3.6	MSSS04	5.7	MSST04	5.5
MS05	3.1	MSS05	3.2	MSSS05	3.8	MSST05	3.3
MS06	3.6	MSS06	3.0	MSSS06	3.8	MSST06	2.6
MS07	3.5	MSS07	3.6	MSSS07	4.0	MSST07	2.8
Wild Type	3.6	Wild Type	3.6	Wild Type	3.6	Wild Type	3.6

mutant out of the entire mutants screened that had higher value than the wild type. Mutant strain with a code MSSS04 from 60 µl EMS treatment showed the highest zone of hydrolysis of 5.7 mm on CMC-agar, while MSSS01, MSSS02, MSSS03, MSSS05, MSSS06 and MSSS07 showed zones of hydrolysis of 3.6 mm and above. The mutants MSST01 to MSST07 developed from 80µl EMS treatment had varied values of zone of clearance. The highest zone of hydrolysis of 5.5mm was recorded from mutant MSST04, followed by mutant MSST01 (3.8mm), MSST02 (4.0mm), while the least value of 2.6mm was obtained from mutant MSST06. The wild type had higher zone of hydrolysis than MSST03, MSST05, MSST06 and MSST07.

The quantitative screening of cells mutagenized with EMS and wild type *B. subtilis* for cellulase production in liquid fermentation is presented in Fig. 1 to 4. Quantitatively, all the mutants developed from 20 µl EMS treatment exhibited specific xylanase activity ranging from 3.4242 µmol/min/mg by MS01 to 16.50 µmol/min/mg by MS05 (Fig. 1). However, wild type had specific activity of 13.67 µmol/min/mg, which was higher than the values exhibited by approximately 86% of the mutants (MS01, MS02, MS03, MS04, MS06 & MS017). A total of seven mutant strains developed from 40 µl EMS treatment produced varied specific xylanase activities with the highest specific activity of 15.97 µmol/min/mg exhibited by MSS07, while the least value of 5.38 µmol/min/mg was produced by MSS05 (Fig. 2). Approximately 86% of the mutants (MSS01, MSS02, MSS03, MSS04, MSS05 & MSS06) had lesser specific activity than the wild type. Two out (MSSS01 & MSSS02) of the seven mutants developed from 60 µl EMS had their cellulase production improved when compared with the wild type, while all the mutants generated from 80 µl EMS had lower specific activities than the wild type (Figs. 3 & 4).

Figure 5 reveals the effect of 0.2% (w/v) glucose supplementation on mutagenized and wild type *B. subtilis* for cellulase production. The mutant strains showed lower specific cellulase activity when compared with wild type. In a medium supplemented with 0.5% (w/v) glucose, mutants MSSS02 & MSSS05 had higher specific cellulase activity than the wild type, while there was repression of specific cellulase activity in others (Figure 6). Similarly, mutants MSSS02 & MSSS05 cultured in a medium supplemented with 1% (w/v) glucose had higher specific cellulase activity than the wild type, while the rest of the mutants had their cellulase activities repressed (Figure 7). The supplementation of enzyme production medium with 1.5% (w/v) glucose caused repression of cellulase production in all the mutants except for MSSS05 which had approximately 34 % higher than the wild type (Figure 8).

Discussion

Improvement in microbial strains is an integral part of process for improved bio-products formation and it often achieved majorly by mutation, a primary source of genetic alteration in parent or wild type. Alteration of microbial genetic makeup can be induced by exposing the organism of interest to a chemical mutagen such as Ethyl methyl sulphonate (EMS) or physical mutagen such as ultraviolet radiation. Mutagenized microbial strains with acquired improved and desirable properties are used

intensively in many industrial processes with a view to reduce cost of production. Thus, recent research efforts are directed towards improving microbial strains via induced mutagenesis for enhanced bio-products formation (Olaniyi et al., 2014; Adeleke et al., 2017).

In this study, exposure of wild type *B. subtilis* to different concentrations of EMS led to an emergence of mutants with varied cellulase activities. In terms of enzyme yield, very few of the mutants had better performance than the wild type. Strains of *B. subtilis* had been improved for industrial enzymes production by the use of chemical mutagens such as EMS, N-methyl- N'-nitro-N-nitroso guanidine and Ethidium bromide (Femi-Ola, 2008; Sangkhakarak et al., 2012; Olaniyi et al., 2015; Park et al., 2017). Femi-Ola (2008) isolated *B. subtilis* from termite and afterward mutagenized it with EMS, and a certain number of the mutants had their cellulase production capacities improved. A mutant from wild type *B. subtilis* represented by P11 developed from EMS treatment had higher endoglucanase production than the wild type (Park et al., 2017). A putative mutant, C1M 26 from wild type *Bacillus* sp. strain C1 after mutagenized with N-methyl- N'-nitro-N-nitroso guanidine (NTG) yielded more cellulase in comparison with a wild type (Sadhu et al., 2014). Similarly, a mutant emerged from NTG treatment yielded higher cellulase than the wild type *Cellulomonas* sp. TSU-03 (Sangkhakarak et al., 2012). Cellulase genes within the improved mutants were suggested to have been affected positively by EMS. However, how cellulase production was triggered in these mutants is yet to be unraveled. We hypothesized that regulatory genes or stability of the nucleotide sequence of mRNA might have been affected by EMS resulting in better cellulase bio-synthesis (Sadhu et al., 2014). Narasimha et al. (2012) and Olaniyi et al. (2014) however attributed improved cellulase bio-synthesis in these few mutants to ease of reconstitution of damaged genes with desired properties. Factors such as damaged DNA sequence and differences in the ability of the mutants to repair damaged genes could account for the variations in cellulase production (Narasimha et al., 2012; Olaniyi et al., 2014).

Cellulase production was repressed in many of the mutants cultured in media supplemented with different concentrations of glucose with few exceptions. Repression of cellulase production in the wild type and mutant strain is expected because glucose, a monosaccharide is already available in enzyme production media and hence there is no need for the secretion of cellulose-degrading enzymes. The preferential utilization of glucose in the presence of other carbohydrates is referred to as catabolite repression and it is regulated by cyclic AMP (Brock and Madigan, 1991). Cyclic AMP interact with allosteric protein known as catabolite gene activation protein (CAP) or cyclic AMP receptor protein (CRP) to form a complex that facilitate prompt binding of RNA polymerase to the promoter site of catabolite sensitive operon which eventually result in the alleviation of the repression state. The secretion of other enzymes capable of utilizing other CHO sources through CRP is impossible as long as the glucose is readily available because its presence lowers the concentration of AMP (Bakare et al., 2005; Olaniyi et al., 2014). This relate to the repression of cellulase production in mutants of *B. subtilis* in current study since they were cultivated in media supplemented with different concentrations of glucose. Repression of cellulase production had been well researched and documented (Kotchoni et al., 2003; Bakare et al., 2005; Zang and Lynd, 2005; Olaniyi et al., 2014). The few mutants with improved cellulase production could be regarded as catabolite insensitive mutants since their cellulase production potentials in the presence of

high glucose was not impaired. The genes that regulate catabolite repression in these catabolite insensitive mutants might have lost their regulatory functions thereby allowing uninterrupted production of cellulase in the presence of repressing glucose (Olaniyi et al., 2014).

The outcome of this investigation indicates that few of the EMS treated mutants produced higher cellulase than the wild type *B. subtilis*. In a bid to overcome the hurdle of catabolite repression, mutants MSSS02 and MSSS05 generated from 60 µl EMS was believed to successfully utilized CMC in the presence of relatively high D-glucose concentration to produce substantial amount of cellulase. Thus, these two mutants (MSSS02 and MSSS05) catabolite insensitive mutants. Conclusively, chemical mutagenesis by EMS caused improvement of cellulase production in few of the mutants by mutation of regulatory genes governing the operation of cyclic AMP or by some mechanisms yet to be known. Therefore, genetic studies on catabolite insensitive mutants might be necessary to reveal altered DNA sequence with improved properties.

Declarations

Acknowledgements

Author acknowledges the technical supports from the Department of Biochemistry, FUTA. This research did not receive any financial support from any funding agency.

Ethics approval and consent to participate

Not applicable

Consent for publication

The author consented for publication in the ABM Express. The current manuscript has been submitted exclusively to this journal and it is not simultaneous considered by another journal. The author declares that no similar work has been submitted to or published by another journal.

Availability of data and materials

Not applicable

Competing interest

It is single-authored manuscript and no conflicts of interest is declared by the author regarding this study.

Funding

This research did not receive any financial support from any funding agency.

Author contributions

Olaniyi, Oladipo Oladipo contributed reagents and materials, carried out the experiment, analyzed and interpreted the data, conceived and designed the experiments and wrote the paper.

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Figures

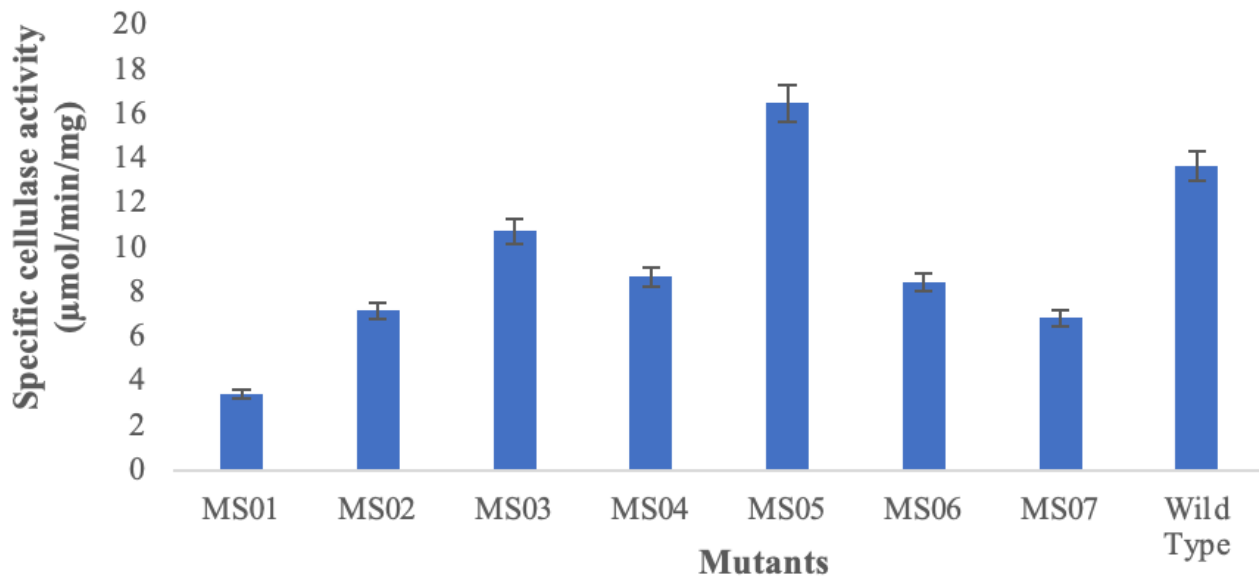


Figure 1

Cellulase production by mutants developed from 20 μl EMS in comparison to wild type *B. subtilis*

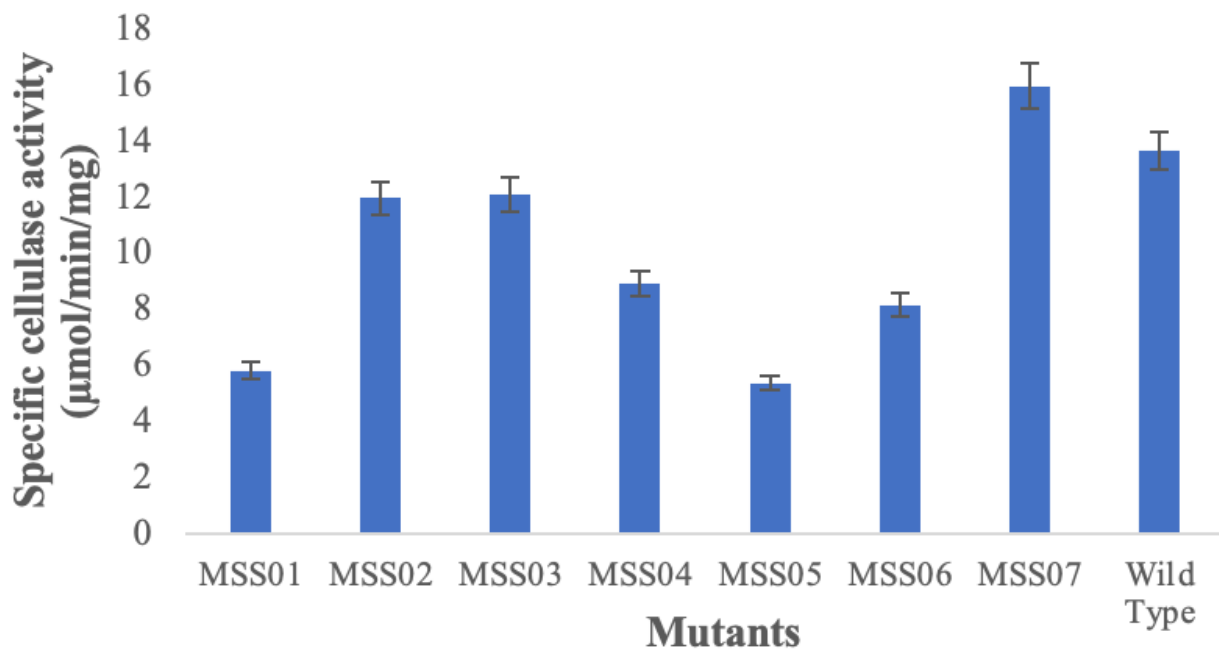


Figure 2

Cellulase production by mutants developed from 40 μl EMS in comparison to wild type *B. subtilis*

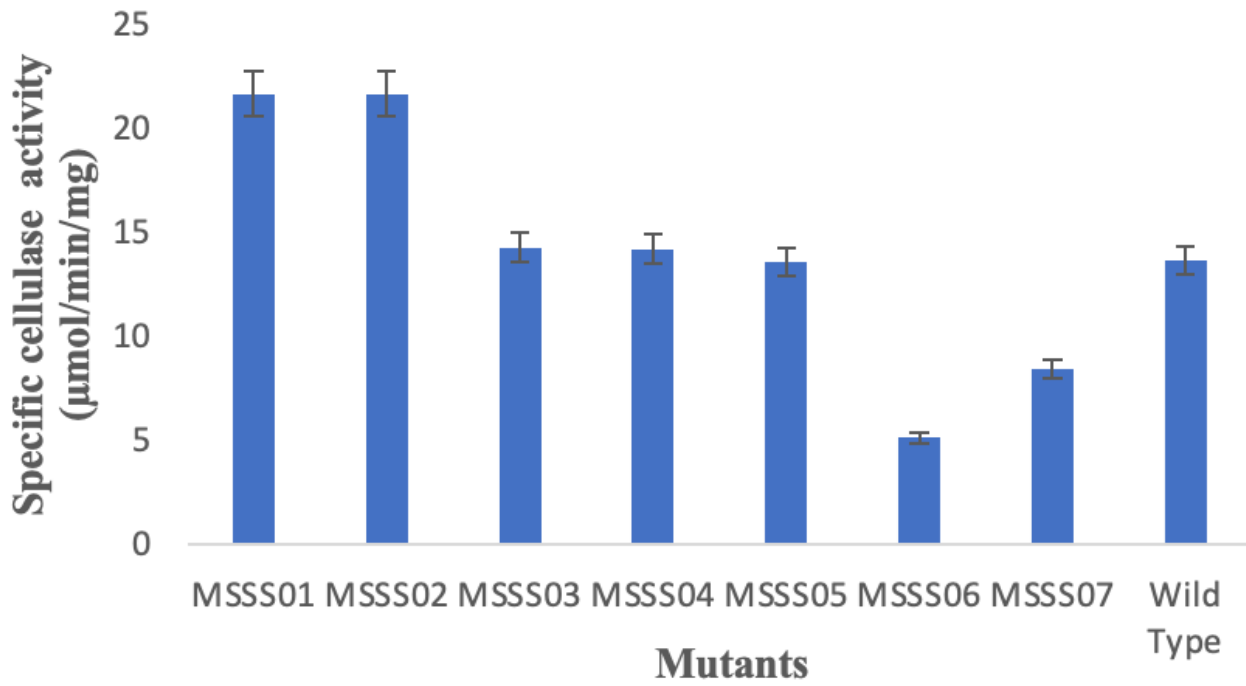


Figure 3

Cellulase production by mutants developed from 60 μl EMS in comparison to wild type *B. subtilis*

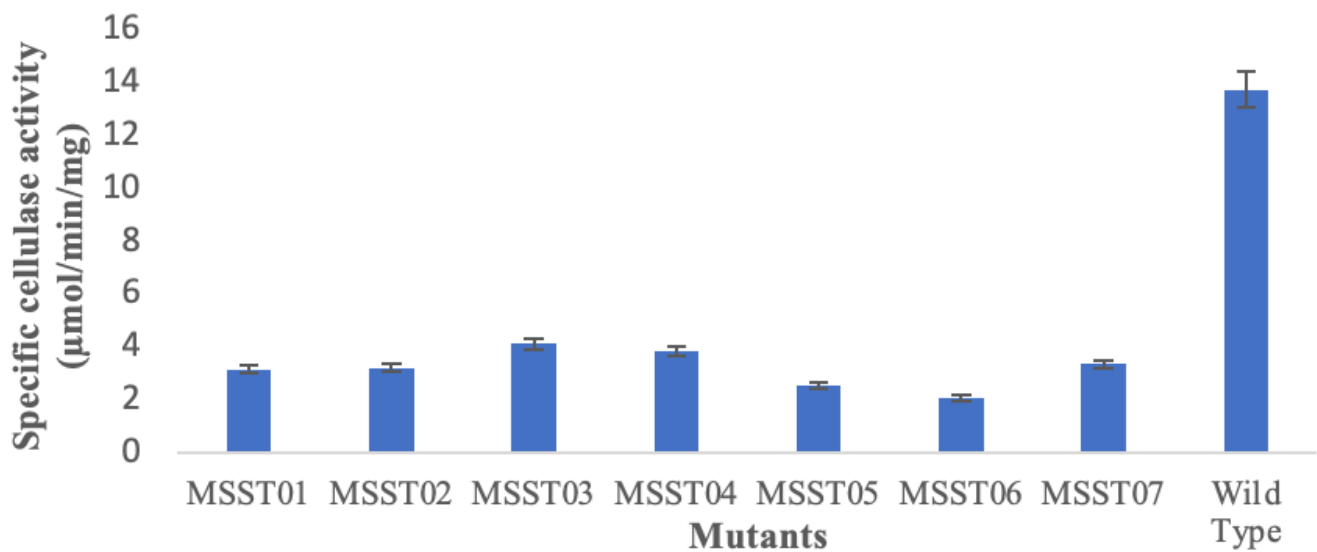


Figure 4

Cellulase production by mutants developed from 80 μl EMS in comparison to wild type *B. subtilis*

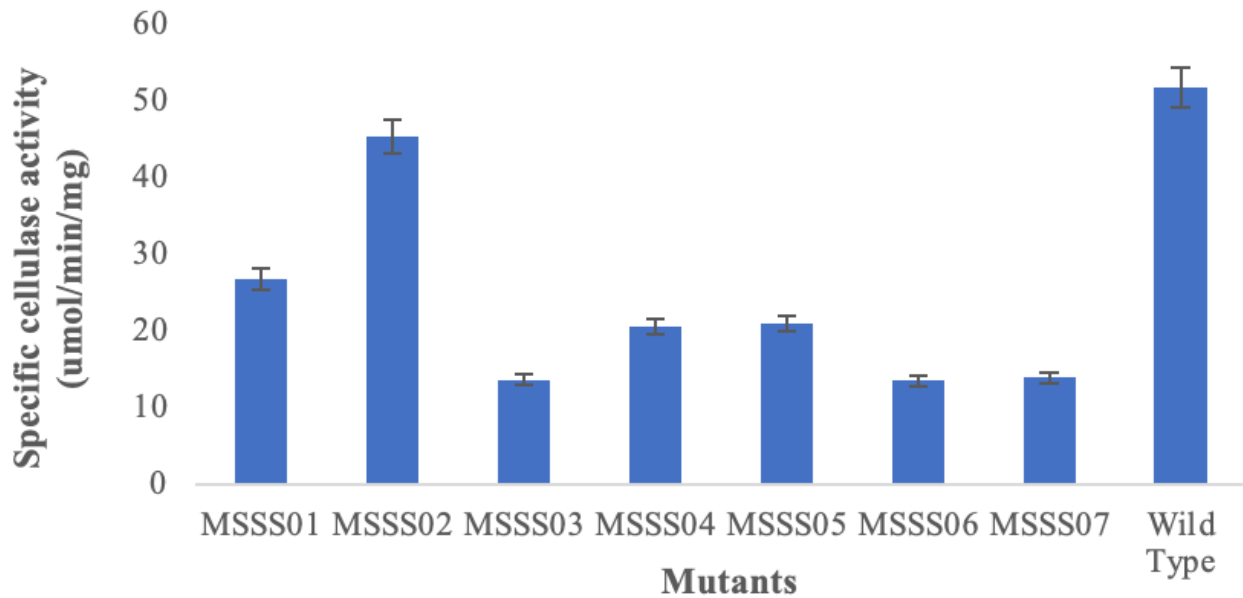


Figure 5

Cellulase production by mutants developed from 60 μ l EMS and wild type *B. subtilis* cultured in 0.2% (w/v) D-glucose

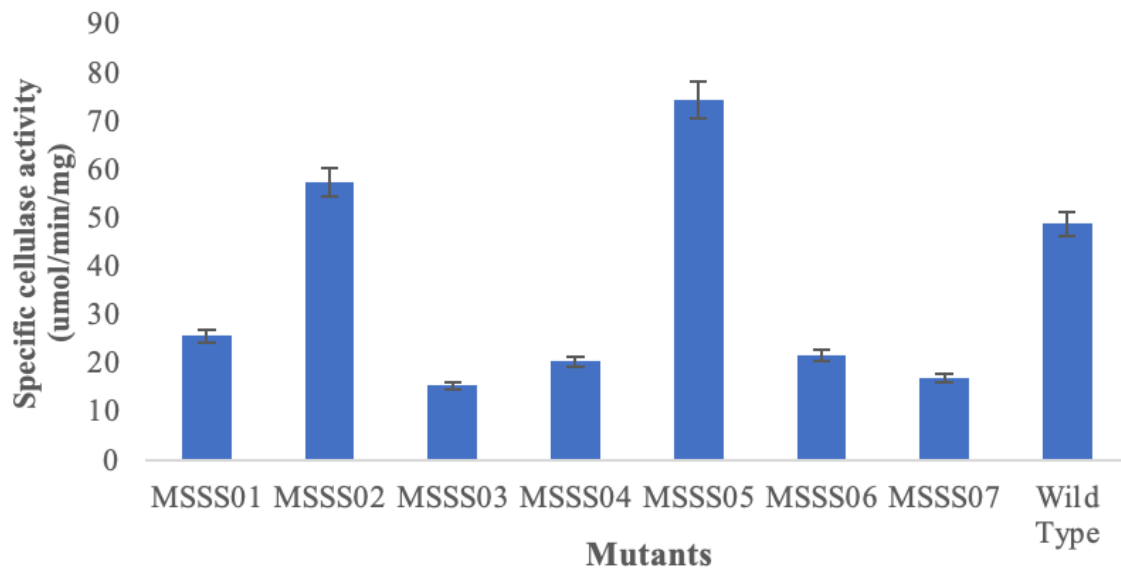


Figure 6

Cellulase production by mutants developed from 60 μ l EMS and wild type *B. subtilis* cultured in 0.5% (w/v) D-glucose

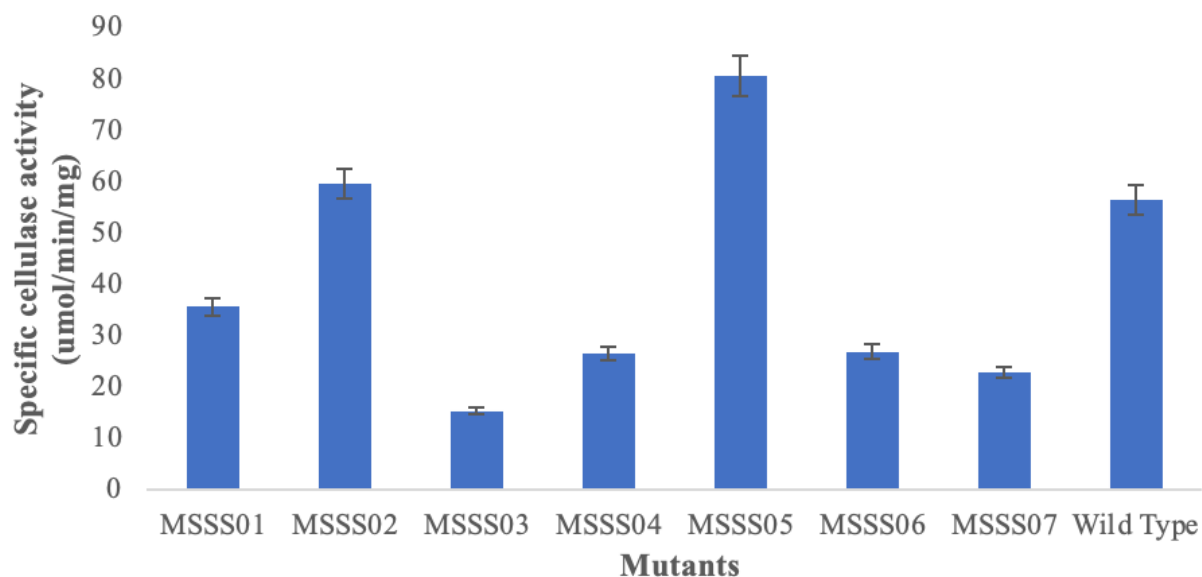


Figure 7

Cellulase production by mutants developed from 60 μ l EMS and wild type *B. subtilis* cultured in 1.0 % (w/v) D-glucose

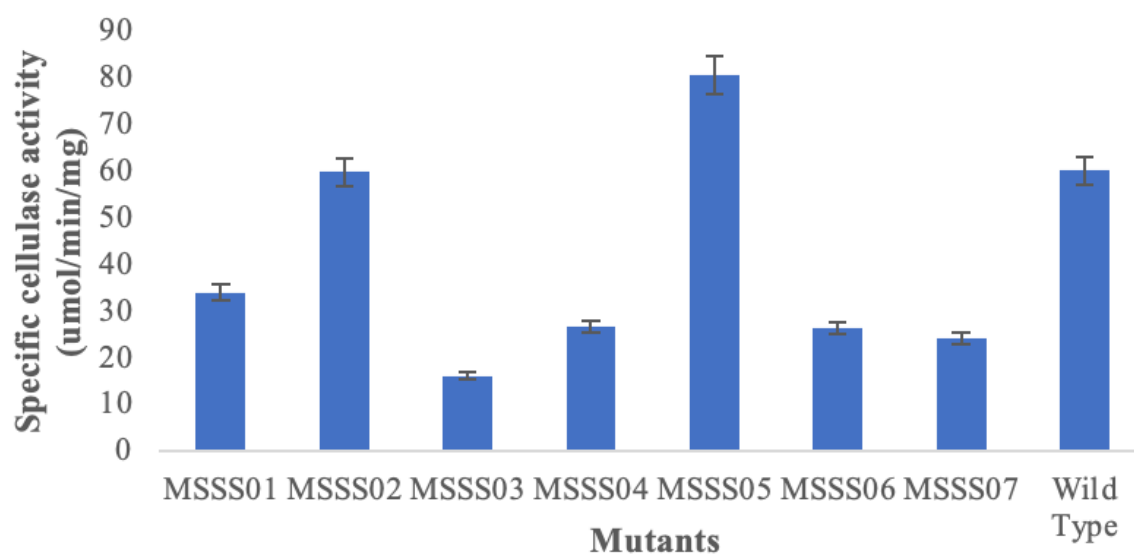


Figure 8

Cellulase production by mutants developed from 60 μ l EMS and wild type *B. subtilis* cultured in 1.5 % (w/v) D-glucose