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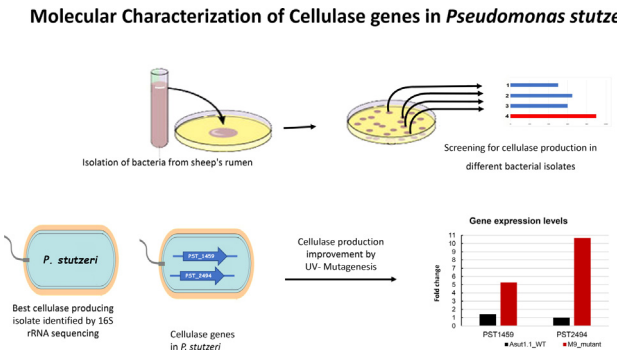
Research Article

Molecular characterization of cellulase genes in *Pseudomonas stutzeri*

Naief H. Al Makishah [a], Ameer E. Elfarash [b,*]

[a] Environmental Sciences Department, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah. P.O. Box: 80208, Jeddah 21589, Saudi Arabia
[b] Department of Genetics, Faculty of Agriculture, Assiut University, Assiut, Egypt

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Cellulose is one of the most abundant natural sources of carbon. In biofuel manufacturing, cellulase is used as an enzyme to hydrolyze cellulose into a fermentable product (glucose). *Pseudomonas stutzeri* is one of the microorganisms found in cattle rumen. The microbiome of the rumen is heterogeneous and known for its potentiality to efficiently hydrolyze cellulose. Recent studies have identified, cloned, and crystallized one of the cellulase genes present in *P. stutzeri*, the A1501 cellulase gene (PST_2494 gene).

Results: This study describes the isolation of cellulase-producing bacteria from sheep's rumen. The highest cellulase-producing isolate was identified as *P. stutzeri* by 16s rDNA sequencing. qRT-PCR was used to measure the cellulase gene expression levels, revealing a higher gene expression of the PST_1459 gene (4 folds) compared to PST_2494 genes. Moreover, cellulase productivity was enhanced by UV irradiation mutagenesis.

Conclusions: Sheep's rumen bacterial isolates were tested for their cellulase productivity, and the highest was identified as *P. stutzeri*. An investigation of the cellulase genes of *P. stutzeri* revealed the presence of an unidentified cellulase gene (PST_1459). A qRT-PCR reaction was carried out to validate and measure the expression levels of different cellulase genes, revealing a higher gene expression of the PST_1459 gene than PST_2494 genes. Moreover, UV irradiation mutagenesis was performed to enhance cellulase productivity. The gene expression tested by qRT-PCR confirmed the enhancement of cellulase productivity in some of the mutants obtained.

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* Corresponding author.
E-mail address: aelfarash@aun.edu.eg (A.E. Elfarash).

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1. Introduction

Plant biomass is the most abundant reserve of carbohydrate and the dominant form of agricultural waste. It mainly consists of three polymeric carbohydrates: cellulose, lignin, hemicellulose, and pectin [1].

A large amount of plant biomass in the biosphere and the rising rate of energy consumption has necessitated the search for alternative methods of biofuel production based on renewable and abundant resources. The use of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of major industrial interest and research, especially with regard to the industrial conversion of biomass to fermentable sugars for transformation to biofuel ethanol [2,3].

Many microorganisms use cellulose as their carbon source by producing extracellular cellulase with different signal peptides for different secretion systems [4]. Herbivores rely on symbiotic gut bacteria to hydrolyze cellulosic material. The rumen contains a complex microbial community consisting of bacteria, anaerobic fungi, methanogenic archaea, and protozoa [5]. As the rumen microbiome is diverse, it is a good medium in which to recover various novel microorganisms that hydrolyze lignocellulosic biomass [6].

P. stutzeri, one of the microorganisms found in cattle rumen, can colonize a variety of environmental niches including oil-contaminated sites, air, crop roots, and human clinical samples. It is a nonfluorescent denitrifying bacterium with a large genome that has about 4,790 coding genes, including CAZymes genes [7].

There is now a need to discover more novel cellulases from various sources [8,9]. Therefore, the objective of this study was to isolate and identify a rumen microorganism that can hydrolyze cellulosic biomass, characterize its cellulase genes, evaluate cellulase gene expression by qRT-PCR, and enhance the cellulase productivity by mutagenesis.

2. Materials and methods

2.1. Sample collection

Sheep rumen samples were collected from Assiut University according to the method described by Abdullah et al. [10]. The samples were preserved in a sterile 50 ml falcon tube before being immediately transferred to a refrigerator, where they were stored at 4°C prior to bacterial isolation.

Bacterial isolation was performed on sucrose-free Czapek's agar medium [11] which was amended with 1% (w/v) CMC as the sole carbon source. Fifty µl of different dilutions from the sheep rumen suspension were plated on the media. The plates were then incubated for two days at 37°C. Colonies which survived on the CMC medium were selected for further studies.

2.2. Screening for cellulase production

Cellulase activity was estimated using two methods; the Congo-red method [12], and then confirmed with the calorimetric DNS test [13].

After incubation for 48 h, CMC agar plates were flooded with 1% Congo-red and incubated for 15 min at 25°C. 1 M of NaCl was used for counterstaining the plates. A clear zone around the growing bacterial colonies indicates its cellulose productivity [12].

The calorimetric method is based on the 3,5 dinitrosalicylic acid (DNS) reduction by sugars [13]. A total of 25 ml of sucrose-free Czapek's broth medium amended with 1% (w/v) CMC was inoculated with the desired isolate and shaken at 100 rpm for 7 d to secrete the cellulase enzyme from the enzyme extract. Following this, 0.8 ml of CMC solution (1 g of CMC dissolved in 99 ml sodium acetate buffer (0.2 M) containing 0.25% calcium chloride) was added to 0.2 ml aliquot of filtered enzyme extract. The mixture was then incubated for 15 min at 55°C in a controlled water bath. Immediately after removing the samples from the water bath, 1 ml of 3,5 dinitrosalycilic acid solution was added. The mixture was heated in a boiling water bath for 5 min and then cooled under running tap water. The total volume of sample was made up to 5 ml with the addition of deionized water. The absorbance of the developed color of samples was measured using a spectrophotometer at a wavelength of 540 nm. The amount of glucose was determined according to a constructed standard curve for glucose made by dissolved increasing amounts of glucose.

2.3. Molecular identification of bacterial isolates by 16S rRNA

DNA sequencing of the 16S rRNA gene was performed to identify the isolate that produced the most cellulase. The sequencing was carried out as follows: the extracted DNA was used as a template in a PCR reaction to amplify the 16S rRNA gene with two universal bacterial primer sets, PS-1 (AGT CGA ACG GCA GCG GGG G) and Ps-2 (GGG GAT TTC ACA TCG GTC TTG CA), [14]. The PCR product was purified and sequenced using an ABI Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, Cal., USA) and an ABI 373 0XL DNA analyser (Applied Biosystems, Foster City, Cal., USA) at the Molecular Biology Research Center (MBRU) at Assiut University. The obtained sequence was compared with sequences available in the GenBank database (NCBI) using a BLAST search, and a phylogenetic tree was constructed with DNAMAN software (version 5.2).

2.4. Enhancing cellulase productivity by mutagenesis

The isolate that produced the highest amount of cellulase was selected to enhance its cellulase production by UV mutagenesis. An overnight bacterial culture was adjusted to reach an OD_{600} of 0.4–0.6. Following this, 1 ml of the culture was spread on Petri plates. Plates were irradiated by a UV lamp at a distance of 10 cm for different durations (0.25, 0.5, 1, 2 and 5 min). Plates were covered and incubated overnight to produce mutants [15]. The production of cellulase by the mutagenized isolates was then carried out as described earlier.

2.5. Quantification of the cellulase gene expression by qRT-PCR

A qRT-PCR experiment was performed to confirm and quantify the expression of the cellulase genes in both wild-type isolates and mutants.

SV Total RNA Isolation System (Promega) was used to extract RNA from an overnight bacterial liquid culture growing on sucrose-free Czapek's medium amended with 1% (w/v) CMC to stimulate cellulase productivity. A DNase treatment using the Max kit from Qiagen was then applied to remove residual genomic DNA. RNA concentration and purity were measured using a Nano Drop Spectrophotometer ND1000 (Nano Drop Technologies). Following this, all RNA samples were diluted to a final concentration of 50 ng/µL and reverse transcription for the RNA samples was performed with the first-strand cDNA Synthesis Kit (Amersham Biosciences, GE Healthcare) using pd (N) random hexamer primers.

A new set of primers was designed according to the sequence of the cellulase genes obtained from the *Pseudomonas* genome database (https://www.pseudomonas.com). The sequences of the primers used are listed in Table 1.

The qRT-PCR was performed at the Molecular Biology Research Center (MBRU) at Assiut University in a Bio-Rad iCycler. The qRT-PCR master mix for each gene was performed using the following

Table 1
Primers used in qRT-PCR quantification.

Gene no.	Primer code	Primer Sequence (3′ → 5′)	Band size	Tm
PST_1459	Ghf5.Cel-Fw	GATGGCAACAAGGTGGGTGC	198	58
	Ghf5.Cel-Rv	GTTGGCCGGAATGGAGAAGC		58
PST_2494	Endo.Cel-Fw	CTGCACGGGGTCAACATCTC	148	60
	Endo.Cel-Rv	CCCAGATGAACGGAAAGCGG		60

reaction components: 12.5 µl iQ SYBR Green Super Mix (Invitrogen), 0.25 µl of each primer (10 µM), and 8 µl nuclease-free water. Following this, 4 µl of the cDNA was added to 21 µl of the master mix. The following PCR protocol was used: initial denaturation (95°C for 10 min), followed by repeated 40 cycles (denaturation: 95°C for 15 s, annealing: 60°C for 20 s, 72°C for 60 s with a single fluorescence measurement), followed by 72°C for seven minutes and cooling to 4°C.

The cycle threshold (Ct) was determined for each gene transcript to quantify the expression of different genes. The Relative Quantification (ΔΔCT) method was utilized to calculate fold change [16]. The gene expression was carried out in triplicate and was normalized with the absolute expression mean values of the reference gene (16s rRNA gene) [17,18].

3. Results and discussion

3.1. Sample collection and cellulase productivity

Two different sheep's rumens were used to isolate bacterial samples. From each sheep's rumen, five different bacterial isolates that were able to perform a visible clear zone on the CMC medium (Fig. 1) were selected to test their cellulase productivity in liquid culture using a DNS test.

The cellulase productivity of the different isolates measured by optimal density is shown in Table 2 which reveal that the production of cellulase measured by optimal density between the different wild-type isolates varied from 0.26 OD in Asut1.1 to 0.04 in Asut1.2.

3.2. Identification by 16S rRNA gene sequencing

The best isolates in terms of the production of cellulase (Asut1.1) were selected for molecular identification. The partial (830 bp) 16S rRNA gene sequence of the Asut1.1 isolate was 100% identical to that of *P. stutzeri* available in the GenBank database.

Table 2
Cellulase productivity in different wild-type isolates measured by optimal density and residual cellulose.

Strain	Optimal density (O.D.)	Residual cellulose (%)
Asut1.1	0.2633	57.67
Asut1.2	0.0400	92.00
Asut1.3	0.1167	76.00
Asut1.4	0.0550	88.00
Asut1.5	0.0783	83.67
Asut2.1	0.1133	76.33
Asut2.2	0.0867	81.33
Asut2.3	0.0967	80.33
Asut2.4	0.1133	76.67
Asut2.5	0.1267	74.33

The ability to secrete large amounts of extracellular cellulase has been extensively investigated. The most commonly studied cellulolytic organisms include the following: *Pseudomonas* [19,20], *Cytophaga*, [21,22], *Bacillus* [23,24], *Serratia* [25,26], *Cellulomonas* [27,28] and *Cellvibrio* [29,30]. The molecular relationship between these best-performing cellulase-degrading bacteria and the Asut1.1 isolate was observed in Fig. 2 which used the 16S rRNA gene sequences to draw the phylogenetic tree. The phylogenetic tree divided the strains into 3 groups. The Asut1.1 isolate (*P. stutzeri*) was located in the 1st group with the following strains: *P. Fluorescens*, *C. mixtus*, and *S. marcescens.* While the 2nd group included the following strains: *B. subtills*, *S. bovis*, and *C. uda.* The 3rd cluster included only *C. hutchinsonii* which showed the lowest genetic similarity.

3.3. Evidence for cellulase productivity of P. stutzeri

Following molecular identification of the isolate that produced the highest amount of cellulase (Asut1.1), which revealed an identical similarity with the *P. stutzeri* 16S rRNA gene sequence, the ability of *P. stutzeri* to secrete extracellular cellulase was confirmed. Similar results were previously obtained by Rastogi et al. [31] and Rattanasuk et al. [19]. Recently, Dutoit et al. [32] identified, cloned, expressed, purified, and crystallized a *P. stutzeri* A1501 cellulase gene (PST_2494, a member of the GH5_5 subfam-

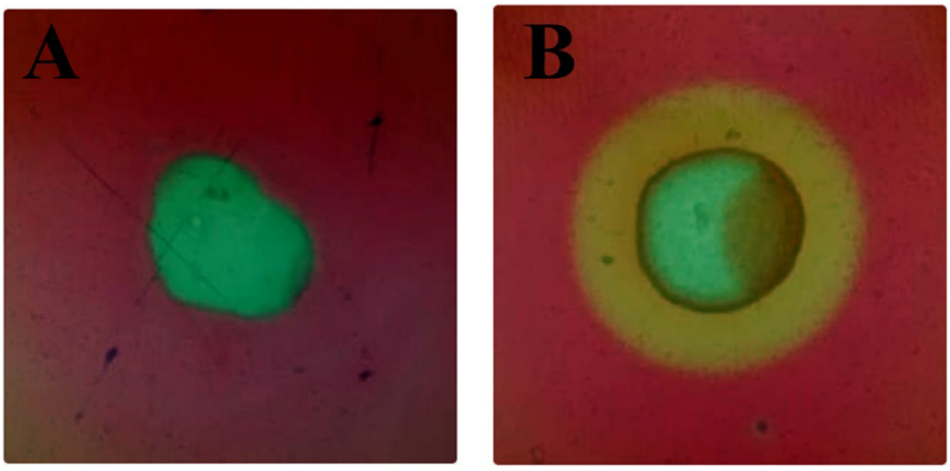

Fig. 1. Cellulase productivity measured by Congo red iodine solution method. (A) Control without CMC degradation; (B) Clear zone on CMC agar plates indicates CMC degradation.

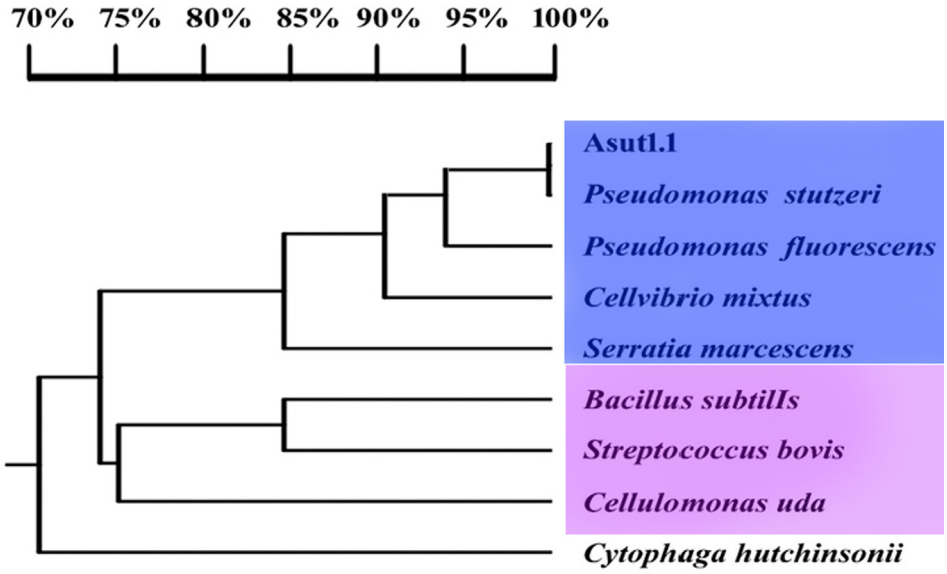

Fig. 2. Phylogenetic tree of the 16S rRNA gene sequence of some cellulase-degrading bacteria and their molecular relationship with the Asut1.1 identified isolate.

ily). No other cellulase genes were identified in *P. stutzeri*. Therefore, the cellulase amino acid sequence of *Enterobacteriaceae* was used to perform a BLASTp investigation to identify other cellulase genes in *P. stutzeri A1501*. The BLASTp revealed the presence of two different candidate genes that could be responsible for cellulase production (Table 3). Based on an alignment analysis, the PST_2494 gene was predicted to encode for an endoglucanase enzyme.

NCBI Multiple Sequence Alignment Viewer (Version 1.21.0) was used to compare the cellulase amino acid sequences from the following different cellulolytic organisms: *Cytophaga* (HHF99360.1), *Bacillus* (WP_158321519.1), *Serratia* (WP_004197531.1), *Streptococcus* (CJK41359.1), and *Cellvibrio* (WP_012486298.1) with the cellulase genes of *P. stutzeri* [PST_1459 (WP_196112182.1) and PST_2494 (ABP80146.1)]. The alignment results showed homology between the cellulases of the different strain sequences (Fig. 3) at a particular domain (the cellulolytic domain), except for the cellulases from *Cellulomonas* (AAC36898.1) and *P. fluorescens* (QOU05665.1); these showed dissimilar cellulase amino acid sequences and may belong to different cellulase families. Based on their mode of action, cellulases can be sub-categorized into endoglucanases, exoglucanases/cellobiohydrolases, and β-glucosidases [33]. Glycoside hydrolases (GHs) are classified into 172 families according to the Carbohydrate-Active Enzymes database (CAZy; https://www.cazy.org) [34].

To predict the cellulolytic domain in the new cellulase PST_1459 gene, the Simple Modular Architecture Research Tool (https://smart.embl-heidelberg.de) was used. The tool compares the protein sequence with sequences of well-studied enzymes and provides clues as to the different domain's locations. The results in Fig. 4 confirm the presence of a cellulase domain at the end of the PST_1459 gene (amino acids 374 – 655). Interestingly, a transmembrane region was found at the beginning of the gene, which may function as a gateway to permit the transportation of cellulase protein across the membrane.

The gene expression of these two genes has not yet been validated. Therefore, RNA from Asut1.1 was isolated and reverse-transcribed to cDNA before being used as a template for a qRT-PCR reaction to quantify the expression of the PST_1459 and PST_2494 genes using the primers listed in Table 1. The results of this study (Fig. 5) confirmed the expression of the two genes in *P. stutzeri*, revealing a higher (four folds) gene expression of the PST_1459 gene than the PST_2494 genes.

3.4. Mutagenesis of Asut1.1 isolate by UV irradiation

Mutagenesis has been used to enhance cellulase production levels in the wild-type isolate that produced the most cellulase (Asut1.1). Mutagenesis was performed for different periods of exposure to UV irradiation. Mutants were selected from surviving bacteria after exposure.

A total of 20 mutants obtained from the mutagenesis of Asut1.1 isolate were screened for cellulase productivity (Table 4). Of these 20 different mutants, four were hyper-producers and 16 were lower producers compared to the wild-type Asut1.1 isolate.

3.5. Gene expression quantification of cellulase genes by qRT-PCR

A real-time PCR (qRT-PCR) experiment was performed to investigate the effect of mutagenesis on enhancing the expression of *P.*

Table 3
Cellulase genes identified in *P. stutzeri* genome and their positions.

Locus	Product	Size	Position		Reference
			Start	End	
PST_1459	glycosyl hydrolase family 5	2079 bp	1565040	1567118	
PST_2494	endoglucanase	1083 bp	2720853	2721935	[32]

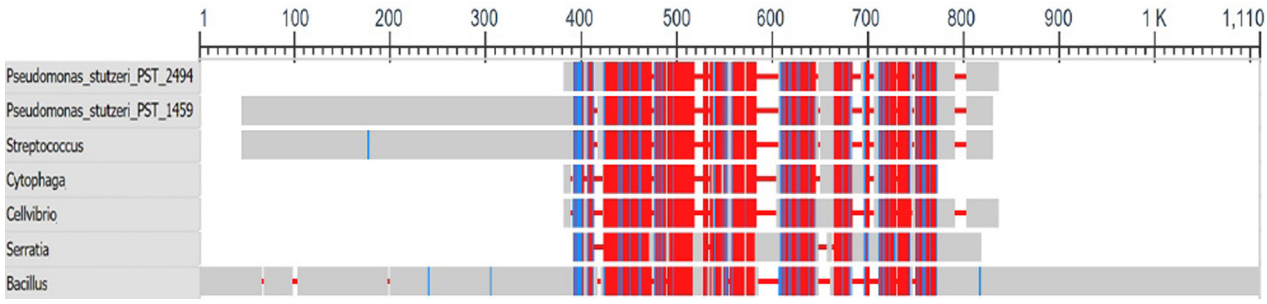

Fig. 3. Alignment of the cellulase protein sequences showing the conserved sequence regions from several organisms. Generated with Multiple Sequence Alignment Viewer 1.21.0.

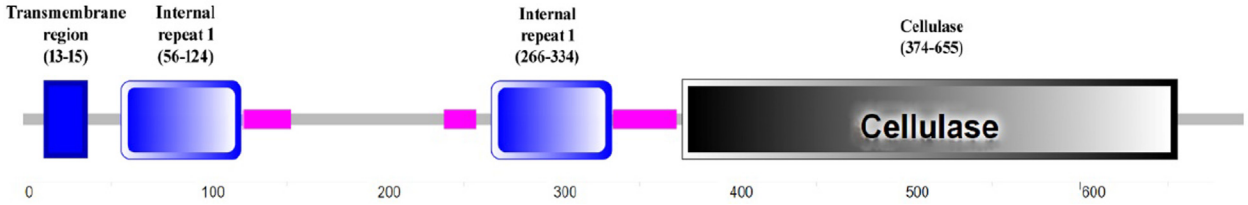

Fig. 4. Different domains of the PST_1459 cellulase protein. Generated with Simple Modular Architecture Research Tool (SMART).

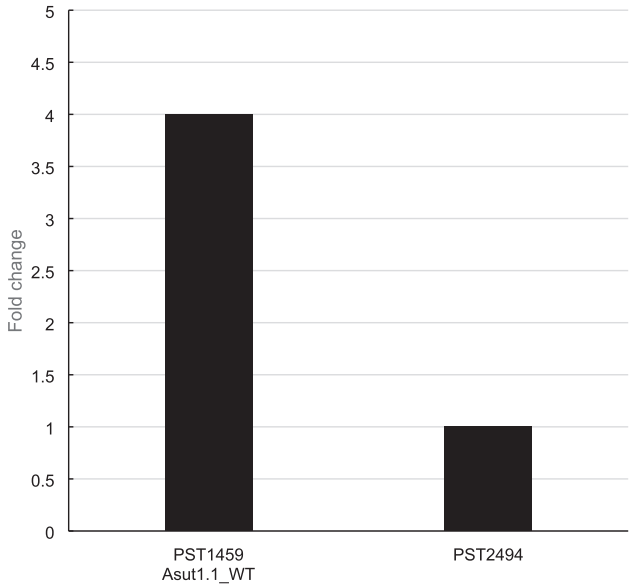

Fig. 5. The fold change in the expression of the different cellulase genes (PST_1459 and PST_2494) present in the wild-type *P. stutzeri* (Asut1.1), confirming the expression of the two genes and a higher (4 folds) gene expression of the PST_1459 gene than PST_2494 genes.

stutzeri cellulase genes. The expression of the two cellulase genes was tested in both **Asut1.1** (the highest wild-type cellulase-producing isolate) and the **M9 mutant** (the mutant that gave the best cellulase productivity), and this was compared to evaluate fold change by qRT-PCR. The comparison of the expression fold change between Asut1.1 and the M9 mutant is represented in Fig. 6.

Table 4
Cellulase Productivity of the 20 mutants produced from the mutagenesis of Asut1.1 isolate.

Isolate	Optimal density (O.D.)	Residual cellulose %
Asut1.1 (control)	0.27	53.53
M1	0.15	71.66
M2	0.11	78
M3	0.22	59.66
M4	0.29	51.33
M5	0.21	62
M6	0.32	48.33
M7	0.04	92
M8	0.16	70.66
M9	0.38	41.66
M10	0.06	86.33
M11	0.31	49
M12	0.22	60
M13	0.17	68
M14	0.18	64.33
M15	0.27	53.33
M16	0.08	82.3
M17	0.07	85.33
M18	0.14	74
M19	0.22	60
M20	0.08	82.33

Labeled cells = Production was in high quantity compared to the wild-type Asut1.1 isolate.

The results in Fig. 6 showed that both genes were expressed in the M9 mutant but with different levels. These results illustrate the importance of the two genes together in degrading cellulose.

Moreover, the gene expression levels in the M9 mutant were higher than the wild-type in both PST_1459 (5 folds) and PST_2494 (10.7 folds) genes, which could be a result of the mutagenesis.

Unlike the wild-type results, the PST_2494 gene showed a higher gene expression than PST_1459 genes after mutagenesis. With these findings, it could be hypothesized that the mutagenesis

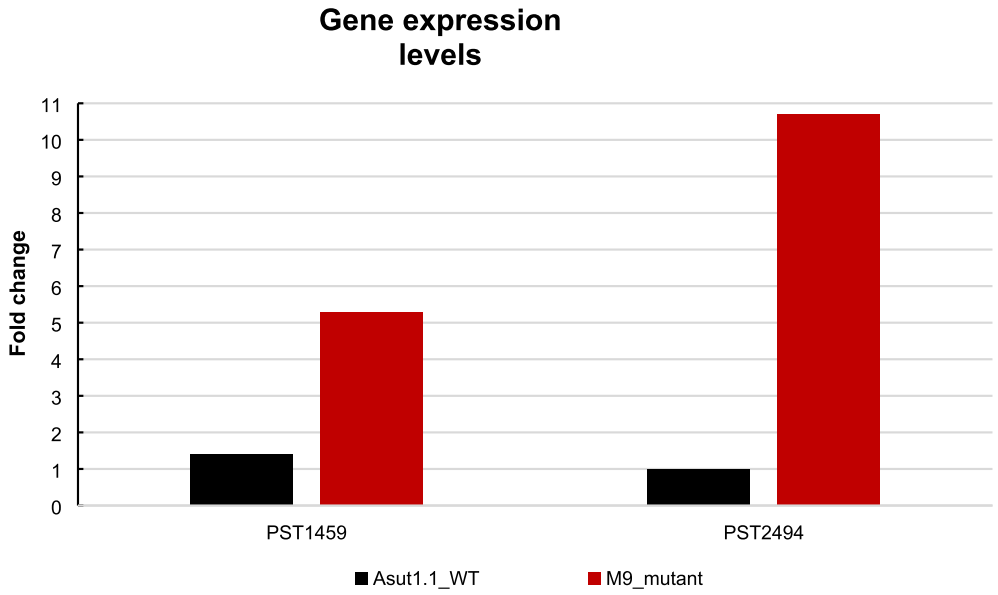

Fig. 6. The fold change in the expression of different cellulase genes (PST_1459 and PST_2494) in both Asut1.1 wild-type isolate and M9 mutant.

caused an overexpression of the PST_2494 gene that increased the cellulase degradation ability in the hyper-producer mutant M9.

4. Conclusions

Sheep's rumen bacterial isolates were tested for their cellulase productivity. The isolate that produced the most cellulase was identified as *P. stutzeri* by 16s rDNA sequencing. A phylogenetic tree was produced to investigate the molecular relationship of the isolated *P. stutzeri* and cellulase-degrading bacteria. An investigation of the cellulase genes of *P. stutzeri* revealed the presence of an unidentified cellulase gene (PST_1459). A qRT-PCR reaction was carried out to validate and measure the expression levels of the cellulase genes, revealing a higher gene expression of the PST_1459 gene than PST_2494 genes. Moreover, UV irradiation mutagenesis was performed to enhance cellulase productivity. The gene expression tested by qRT-PCR confirmed the enhancement of cellulase productivity in some of the mutants obtained.

Ethical approval

The study protocol was approved by the MBRSI-Research Ethics Committee, Assiut University, Egypt with the approval number: AGR-21-52-R.

Conflict of interest: All authors declare no conflicts of interest in this paper

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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