Effect of CMC and MCC as Sole Carbon Sources on Cellulase Activity and *eglS* Gene Expression in Three *Bacillus subtilis* Strains Isolated from Corn Stover

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Cellulolytic activities in *Bacillus subtilis* have been demonstrated and it is known that the *eglS* gene encodes an endoglucanase that could play a key role. Three *Bacillus subtilis* strains (RZ164, RS351, and RS273) isolated from corn stover with contrasting cellulase activity were examined in this work. The aim was to analyze the influence of *eglS* gene on the ability of bacteria to grow on a liquid medium supplied with carboxymethyl cellulose (CMC) or microcrystalline cellulose (MCC) as sole carbon sources. All strains displayed similar growth in CMC medium and comparable exoglucanase and endoglucanase activity. However, the expression of *eglS* did not correlate among strains. On the other hand, when MCC was the carbon source tested, the growth of RS351 was higher than that obtained by RZ164 and RS273 strains. This behavior could be related to the level of cellulase activities displayed by this strain. Besides, *eglS* expression was higher in RS351 strain, suggesting a direct participation of this enzyme when the carbon source is MCC. Taken together, *eglS* could be involved in different roles exerted by these strains on either exo- or endoglucanase activity and under either substrate. The enzymes described here could be considered good alternatives for biomass conversion.

Keywords: Biofuel; Bacillus subtilis; Endoglucanase; Carboxymethylcellulose; Microcrystalline cellulose; qRT-PCR

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INTRODUCTION

Currently, there is an increasing demand for cellulases for various applications, the most important being for the bioconversion of lignocellulosic biomass for ethanol production (Srivastava *et al*. 2015). Cellulases are the third largest industrial enzyme worldwide by dollar per volume, because of their wide spectrum of applications, such as cotton processing, paper recycling, juice extraction, as detergent enzymes, and animal feed additives (Singhania *et al*. 2010). Considering the importance of ethanol production, cellulases could become the largest enzyme volume used by the fuel industry.

Cellulases are enzymes that hydrolyze the β-1,4-glucan linkages in cellulose producing as primary products, glucose, cellobiose, and cello-oligosaccharides, which are suitable substrates for bioethanol production. These enzymes have been extensively studied, and they form a complex including endo-β-1,4-glucanases (EC 3.2.1.4), exo-β-

1,4-glucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Endoglucanases produce nicks in the exposed cellulose polymer, which produces both reducing and non-reducing ends. Exoglucanases or cellobiohydrolases act on these ends to produce cello-oligosaccharides and cellobiose units. To fully hydrolyze cellulose, β-glucosidases cleave the cellobiose, producing glucose units (Sharma *et al*. 2016).

Cellulases are produced by a wide variety of fungi and bacteria; however, it is easier to purify bacterial cellulases than fungal cellulases (Maki *et al*. 2009). In addition, since the generation time of bacteria is short and they can be easily grown in synthetic-culture media to obtain high cell biomass using inexpensive carbon and nitrogen sources, bacteria have become excellent organisms for the production of secreted enzymes used in the bioconversion industry (Keshk 2014).

In particular, several species of the genera *Bacillus* produce cellulases, including strains of *B. cereus*, *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens,* and alkaliphilic *Bacillus* (Horikoshi 1997; Bischoff *et al*. 2006; Nurachman *et al*. 2010; Yan *et al*. 2011; Asha and Sakthivel 2014). These enzymes have shown properties of industrial interest such as halotolerance and stability in the presence of surfactants and metal ions (Zhu *et al*. 2011).

A wide variety of *Bacillus* strains have been characterized at the biochemical level; authors have detected cellulase activity in *Bacillus* strains growing in carbon sources such as carboxymethyl cellulose (CMC, a soluble and amorphous cellulose) (Bano *et al.* 2013; Orehek *et al*. 2013), microcrystalline cellulose, *e.g.* Avicel® (Di Pascua *et al*. 2014), and sugarcane bagasse (Bano *et al*. 2013). The cellulase activity is regulated, at least in part, at the genetic level, with the participation of genes that encode for endoglucanase, exoglucanase, and β-glucosidase (Sukumaran *et al*. 2005).

In silico analysis of the *B. subtilis* 168 genome revealed the presence of genes that code for an endoglucanase; nevertheless, there was no candidate gene related to exoglucanase activity (Barbe *et al*. 2009). To explain the presence of both endoglucanase and exoglucanase activities, Han *et al*. (1995) and Zhao *et al*. (2012) have reported that *B. subtilis* produces a bifunctional enzyme, acting both as endoglucanase and exoglucanase; however, the authors did not show any genetic or experimental evidence. The *eglS* gene encodes for a thermostable endoglucanase in *B. subtilis* 168, which hydrolyzes carboxymethyl cellulose and MCC (Santos *et al*. 2012); however, additional molecular studies on this gene's function are still not available. In this study, we analyzed the cellulolytic activity and the expression of the *eglS* gene, under different carbon sources, by three *B. subtilis* strains, which were isolated from corn stover. The goal of the present work was to establish a link between the endo- and exo-glucanase activities with the expression of gene *eglS*.

All three strains were able to use either CMC or MCC, but one strain (RS351) showed a higher growth rate than the strains RZ164 and RS273 when grown with MCC. When using CMC as a carbon source, *eglS* expression showed a particular pattern for two of the three strains, which could indicate a differential role of *eglS* among the analyzed strains. However, when using MCC as the sole carbon source, the rate of *eglS* expression showed a peak before the cellulase activity in all three strains, but only showed a second expression increment in RS351, which was coincident with the latter increment in both endo- and exo-glucanase activities. These results suggest that *eglS* could play major but different roles related to cellulase activity among the three *B. subtilis* strains.

EXPERIMENTAL

Microorganisms and Culture Conditions

The strains RS351, RS273, and RZ164 with cellulolytic potential were obtained from a rhizosphere and corn stover bacteria collection isolated from agricultural fields in Sinaloa, Mexico. These strains were selected based on their ability to hydrolyze a mineral medium supplemented with 1% CMC (w/v) as sole carbon source and Congo red as indicator, described by Teather and Wood (1982). The isolates were identified as *B. subtilis* after sequencing and comparing 1200 bp of the 16S gene against the NCBI and RDP (Cole *et al*. 2003) databases (data not shown).

To evaluate the effect of the carbon source in cellulolytic activity, these strains were grown at 30 °C and 200 rpm in 500-mL flasks containing 100 mL of liquid medium prepared as follows: 1 g/L KH_2PO_4, 0.70 g/L $MgSO_4$, 0.5 g/L NaCl, 0.70 g/L $FeSO_4$, 0.30 g/L NH_4NO_3, and 0.30 g/L $MnSO_4$, supplemented with (10 g/L) of either CMC or MCC (Avicel®) (Crawford and McCoy 1972). To evaluate the growth in each carbon source, colony forming units (CFU) were counted on LB agar plates at 0, 24, 48, 72, 96, and 120 h post-incubation.

Enzymatic Activity

For cellulolytic activity measurements, 5 mL of the culture medium were collected at 0, 24, 48, 72, 96, and 120 h of incubation. Cell-free medium was collected after centrifugation at 6000 *g* per 20 min. Enzyme activity was measured according to Zhao *et al.* (2016) with some modifications. Endoglucanase activity was determined by incubating 900 µL of CMC (1%) with enzymatic extract to a final volume of 1.1 mL containing 0.1 M acetate buffer, pH 6.0, at 50 °C for 50 min. Exoglucanase activity was evaluated by incubating 12.5 mg of filter paper and 800 µL of enzyme extract in 1 mL of reaction mixture containing 0.1 M acetate buffer pH 6, at 50 °C for 50 min. The released reducing sugars were estimated by the DNS method (Miller 1959). The enzymatic activities were calculated by the formula reported by Silveira *et al*. (2012) and expressed in international units (IU) per milliliter, considering a unit as the amount of enzyme that releases 1 µmol of glucose per min.

RNA Extraction and RT-qPCR

RNA was purified using TRIzol reagent (Invitrogen, NY, USA). The cDNA was synthesized using the Superscript III (Invitrogen) with random hexamers following manufacturer's instructions. The oligonucleotides used for *eglS* expression (Fwd-GTTCACACGGATTGCAATGG, rev-TACATCGCTGCACGGAAAAC) were designed with Primer3 plus (Untergasser *et al*. 2012) using the *eglS* gene from *B. subtilis* 168 (Barbe *et al*. 2009). The *rpoB* gene was selected as a reference gene for normalization (Ho *et al.* 2011). In order to evaluate the reproducibility of the reaction, a calibration curve was prepared using 1000, 100, 10, 1, and 0.1 ng of cDNA from a control sample and 200 nmol/L of each oligonucleotide from the reference gene according to Nolan *et al*. (2006). Each 10-µL RT-qPCR mixture contained 5 µL of SYBR green master mix, 1 µL of cDNA, 1 µL each of 5 µM forward and reverse oligonucleotides, and 2 µL of ultrapure water. Real-time RT-qPCR was performed on a Rotor-Gene Q (Qiagen, Hilden, Germany). Cycling conditions were as follows: initial denaturation at 95 °C for 5 min and 45 cycles of 95 °C for 5 s, 60 °C for 10 s, and a temperature variation step of 60 to 90 °C to determine the melting curve. For data analysis, 0 h was selected as a control. The

comparative threshold cycle 2-ΔΔCt value was used as the method for the relative mRNA expression (Salvioli *et al*. 2012).

Statistical Analysis

Three biological and technical replicates were conducted for each experiment. Data were analyzed by analysis of variance (ANOVA) using SAS 9.0 software (SAS Institute, NC USA).

RESULTS AND DISCUSSION

Growth Curve

Growth of RZ164, RS351, and RS273 was monitored at 0, 24, 48, 72, 96, and 120 h post-incubation in mineral medium with CMC or MCC as sole carbon source (Fig. 1).

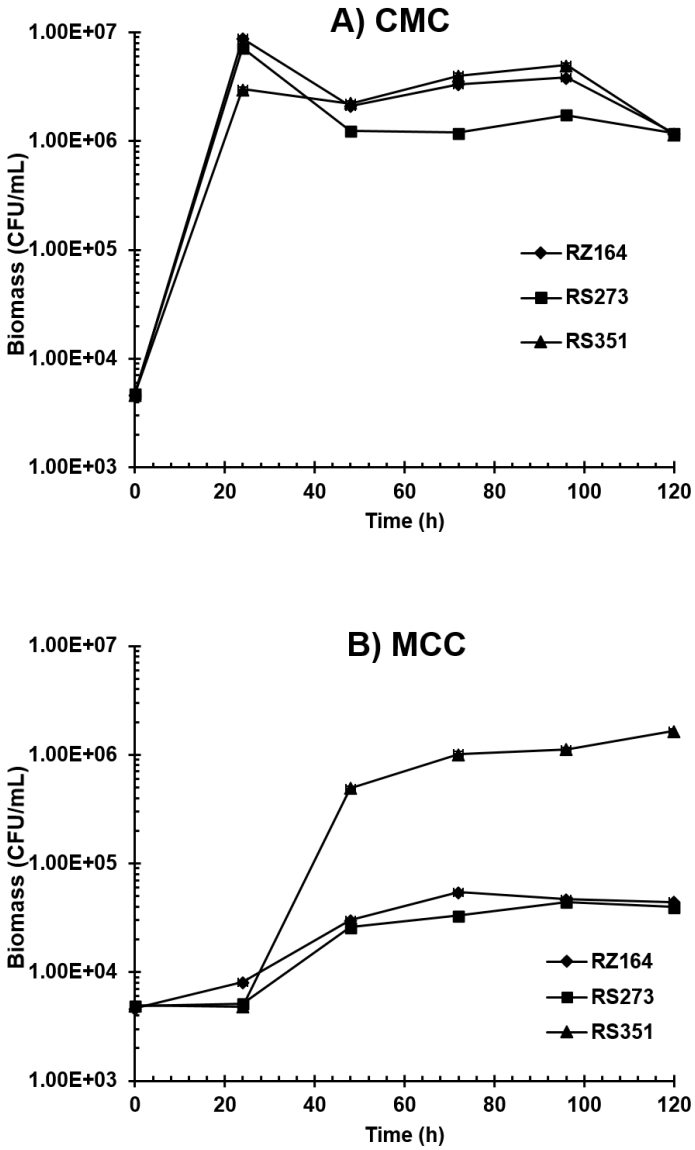

Fig. 1. Growth curve of *B. subtilis* strains RZ164, RS351, and RS273 under A) CMC and B) MCC as sole carbon sources.

In CMC, the stationary phase was reached at 24 h, whereas it was reached at 48 h in MCC. It is worthwhile noting that MCC had a positive effect on the growth rate as well as on the higher biomass of RS351 (1.66×10^6 CFU/mL). This was particularly evidenced at 48 h.

Cellulolytic Activity

To explain the growth achieved in CMC and MCC, endoglucanase and exoglucanase activities were measured. When CMC was used as the carbon source, endoglucanase activity was detected after 24 h, with maximum values between 48 and 96 h for all three strains (Fig. 2A). Notoriously, exoglucanase was also detected for all three strains at 24 h of incubation with maximum values at 96 and 120 h (Fig. 3A); the present activity data are similar to a previous report (Kim *et al.* 2012).

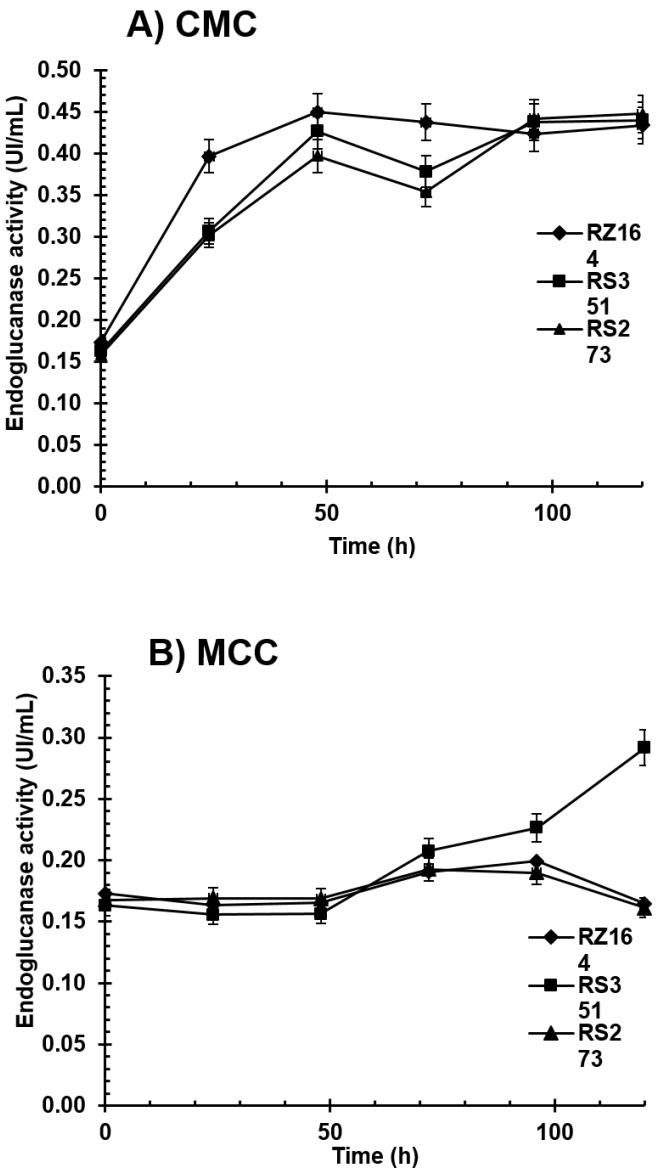

Fig. 2. Endoglucanase activity of *B. subtilis* strains RZ164, RS351, and RS273 with A) CMC and B) MCC as the sole carbon sources**.** Bars indicate standard deviations. UI indicates international units expressed as the amount of enzyme that catalyzes the release of 1.0 µmol of glucose per minute.

When MCC was used as the sole carbon source, endoglucanase activity increased after 48 h, reaching the maximum values at 96 and 120 h (Fig. 2B), whereas exoglucanase activity was detected only after 72 h (Fig. 3B). When compared to RZ164 and RS273, RS351 presented significantly higher endo- and exo-glucanase activities. The RS351 maximum activity at 120 h was lower than previously reported (exoglucanase activity of 1.5 IU/mL. Oliveira *et al*. 2014), for a *Bacillus sp*. strain; however, different experimental conditions were employed for detecting exoglucanase activity. In the literature, it has been reported that members of the *Bacillus* genus do not degrade microcrystalline cellulose (Kim 1995); however, the present results and those of others (Mawadza *et al*. 2000, Oliveira *et al*. 2014) have demonstrated that *B. subtilis* presents exocellulase activity when the bacterium is cultivated in a medium supplemented with MCC as the carbon source.

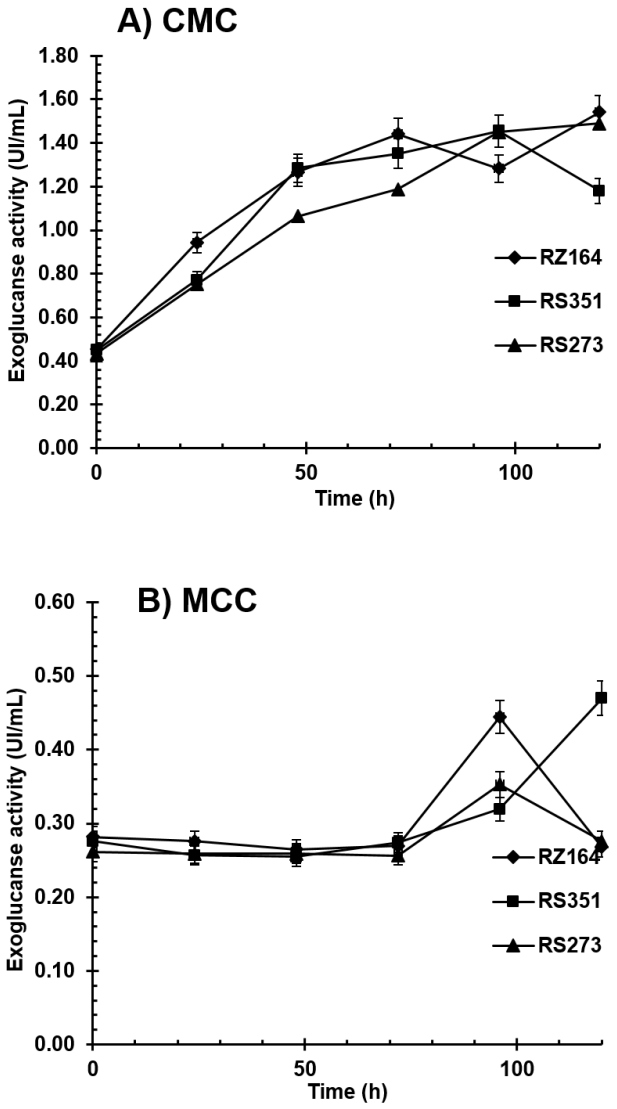

Fig. 3. Exoglucanase activity of *B. subtilis* strains RZ164, RS351, and RS273 with A) CMC and B) MCC as the sole carbon sources. Bars indicate standard deviations. UI indicates international units expressed as the amount of enzyme that catalyzes the release of 1.0 µmol of glucose per minute.

eglS Expression in *Bacillus* strains grown in CMC or MCC

To gain insight into the role of *eglS* gene in cellulase activity and growth of *B. subtilis* cultured with CMC or MCC as sole carbon sources, its relative expression was evaluated by RT-qPCR. In CMC, the strains RZ164 and RS273 exhibited similar *eglS* expression patterns by showing an increase in transcript levels at 24 h, followed by a decrease at 48 h, and again an increase at 72 and 96 h. Although no clear conclusion can be drawn, the first peak in gene expression at 24 h could be directing one enzymatic activity and the second peak activating other activity, as suggested previously (Han *et al*. 1995 and Zhao *et al*. 2012). Regarding RS351, even though its growth rate, endo and exoglucanase activity were similar, no correlation existed with its *eglS* expression, as this was quite low, with a slight increase in expression at 24 h (Fig. 4A).

On the other hand, when the strains were grown in MCC, RZ164 and RS273 showed similar *eglS* expression, with an induction at 48 h and a subsequent decrease at 72, 96, and 120 h (Fig. 4B), whereas the RS351 strain showed an increase in the *eglS* transcript level at 48 h, then a decrease at 72 h, followed by a second increase thereafter.

Despite the fact that in CMC, the strain RS351 grew at the same levels as RZ164 and RS273 with equivalent endo- and exo-glucanase activities, *eglS* expression was significantly lower in RS351 (Fig 4A). Considering that the enzymatic activities were similar among all three strains, it would be expected that they would present similar *eglS* expression levels. The contrasting expression levels among strains growing in CMC suggest that either *eglS* is not directly related to CMC degradation or that it has contrasting roles among these strains. Several authors have observed differences in cellulase activity among members of the *Bacillus* genus (Afzal *et al*. 2010; Kim *et al*. 2012). While there are no studies supporting differences in cellulase expression patterns in *Bacillus*, the increase in transcript levels at 24 h could have triggered the detected cellulase activities.

Regarding the behavior observed with MCC, RZ164 and RS273 presented similar *eglS* expression patterns, showing an induction at 48 h and a subsequent decrease at 72, 96, and 120 h (Fig. 4B). Observed *eglS* expression levels at 48 h could be supporting either endo- or exo-glucanase protein synthesis and account for the observed activity (Fig. 2B and 3B). The expression of *eglS* in the RS351 strain, which showed higher levels of both endo- and exo-glucanase at 96 and 120 h, was also contrasting. An increase in the *eglS* transcript level was observed at 48 h, which decreased at 72 h, and finally increased again at 96 and 120 h, whereas *eglS* expression in RZ164 and RS273 remained low (Fig. 4B). Similar results were described by Di Pascua *et al*. (2014), where the expression of *bglC*, an orthologue gene in *B. amyloliquefaciens* growing on MCC as the sole carbon source, increased at the lag phase, decreased at the exponential phase, and finally increased at the stationary phase. The *eglS* expression trend observed in RS351 was coincident with the detected endo- and exo-glucanase activities on MCC as the sole carbon source at 120 h (Fig. 2B and 3B). Differences in RS351 were also observed when growth was compared (Fig. 1B), showing a higher growth rate. It is possible that the high expression of *eglS*, as well as cellulose activity (either endo or exo-glucanase or both), influences the higher cell growth observed at later time points; however, this would require further confirmation. Here, the enzymatic activity and gene expression did not correlate always. Wei *et al*. (2012) reported that the expression pattern and cellulose activity took place in a coordinated way, which allowed the overall efficiency of cellulose degradation, although it is known that enzymatic activity and gene expression do not always show the same behavior.

To evaluate whether *eglS* gene sequence could explain differences in cellulase activities or expression among the analyzed strains, DNA of *eglS* from RZ164, RS273 and RS351 was fully sequenced. No significant differences were found at the nucleotide level (sequences shared a 97% identity, data not shown). Further experiments are required to fully understand the reason why the expression of *eglS* in RS351 with CMC decreases after 24 h and increases at 96 h with MCC.

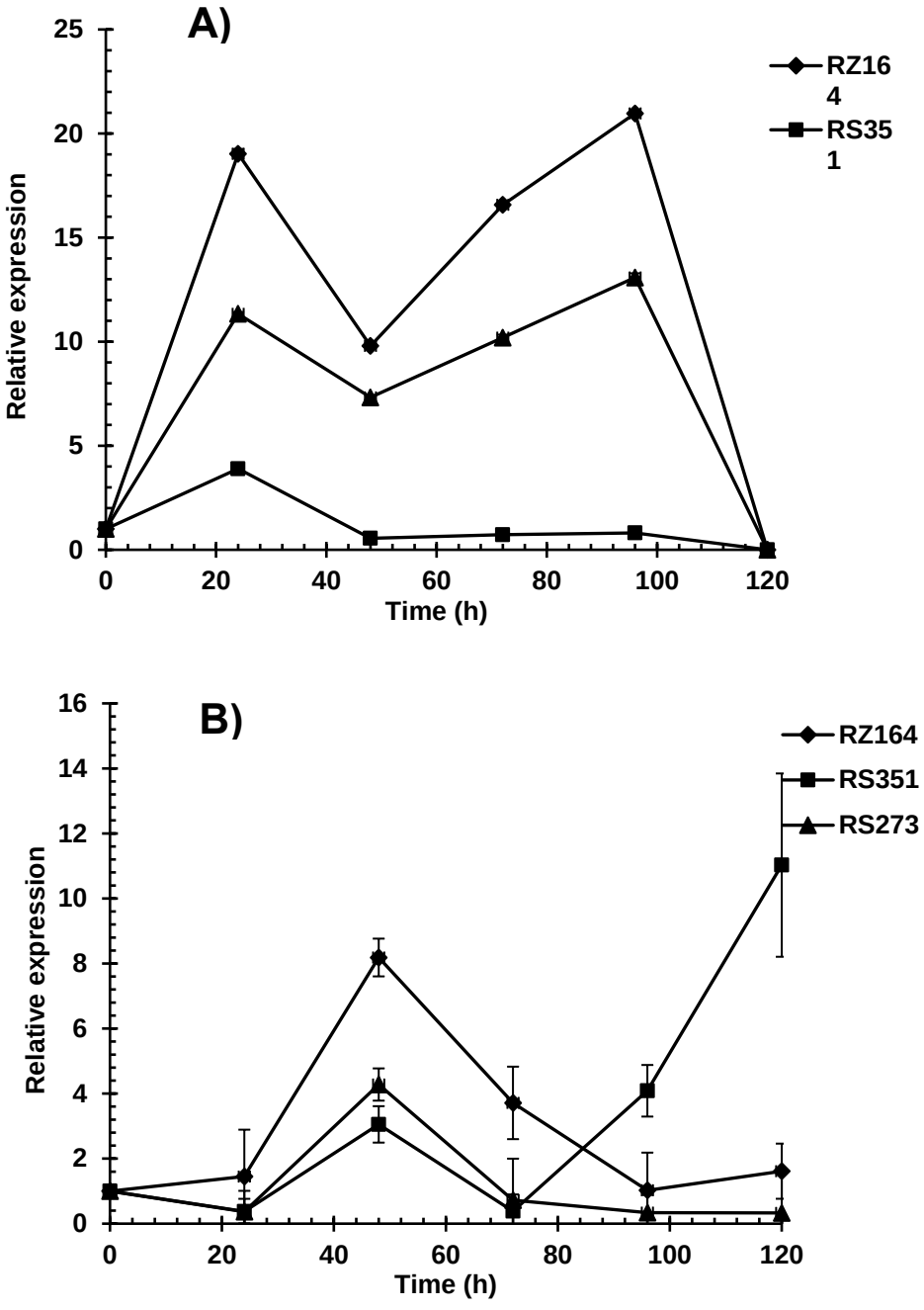

Fig. 4. Transcript level of *eglS* gene of *B. subtilis* strains RZ164, RS273, and RS351 under A) CMC and B) MCC as the sole carbon sources. Error bars indicate the standard deviation.

CONCLUSIONS

There were differences in growth, cellulase, and *eglS* expression among the evaluated strains. Although all three strains exhibited the ability to grow with either CMC or MCC as the sole carbon source, only RS351 presented a higher growth rate when cultivated in MCC.

On MCC, cell growth, cellulase activity, and *eglS* expression were coordinated for each strain, which would confirm the role of *eglS* on endoglucanase and exoglucanase activity in cellulose degradation. On the other hand, as no correlation was observed between *eglS* expression and cellulose activities when strains were grown in CMC, it is possible that the participation of *eglS* on CMC degradation and cellulase activity could be strain-specific or is being regulated by other factors different from its genetic expression.

Finally, the strains reported here showed good cellulase activities in short times, acting on both amorphous and microcrystalline cellulose; hence, they may prove advantageous in industrial applications.

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