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# Purification and characterizations of a novel recombinant *Bacillus velezensis* endoglucanase by aqueous two-phase system

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## Abstract

**Background:** Cellulases played an important role in the production of bioenergy and bio-products. Cellulases from bacteria with some special characteristics drew great attention due to its fast growth speed, wide adaption to harsh environment, and production of multi-function cellulases.

**Results:** An endoglucanase gene *egls* from *Bacillus velezensis* A4 was cloned and expressed in *Escherichia coli* BL21 (DE3). The recombinant enzyme Egls was partially purified using aqueous two-phase system. The highest recovery rate of the enzyme was 90.39% at PEG 4000 (25% w/w), phosphate buffer 8.08% (w/w) (pH 6.0), and NaCl (5% w/w). The enzyme molecular weight was 55 KD estimated by zymogram. The optimal pH and temperature of recombinant enzyme Egls were pH 6.0 and 55 °C, respectively. The enzyme was stable at pH range of 5.0–7.0 at 55 °C for 60 min. The enzyme exhibited $K_m$, $V_{max}$, $K_{cat}$ values as 63.38 mg/ml, 55.6 mg/min, and $3.93 \times 10^3$/S, respectively. The addition of 10 mM of $Mg^{2+}$, $Mn^{2+}$, or 5% (w/w) of Triton-X 100 in the reaction system enhanced the enzyme activity significantly. The enzyme showed both endoglucanase and exoglucanase activity.

**Conclusions:** An endoglucanase gene egls from *B. velezensis* A4 was cloned and expressed in *E. coli* BL21 (DE3). The recombinant enzyme Egls was purified by aqueous two-phase system and characterized. The enzyme can be applied for the efficient pretreatment of lignocellulosic biomass for bioenergy and bio-products production.

**Keywords:** Purification and characterizations, Endoglucanase, *Bacillus velezensis*, Aqueous two-phase system

## Background

Cellulose, hemicellulose, and lignin are the most abundant, cheap, and renewable nature resources in the world (Basu 2013). Cellulose accounts for about 1/3 of the lignocellulosic biomass (Sun and Cheng 2002). Hydrolysis of cellulose to produce reducing sugars using lignocellulosic biomass is extremely important in the production of renewable bioenergy and other bio-products (Pérez et al. 2002; Yang et al. 2014).

The biodegradation of cellulose was mainly conducted by various kinds of cellulase (Endoglucanase, exoglucanase, and β-glucosidase) (Garvey et al. 2013). Endoglucanases randomly cut the cellulose polysaccharide chain, generating various lengths of oligosaccharides. Exoglucanases act on the ends of cellulose polysaccharide chains, producing either glucose or cellobiose as major products. β-glucosidases hydrolyze cellobiose from non-reducing end to produce glucose (Rabinovich et al. 2002).

Cellulases could be produced by various fungi and bacteria. Fungi have the ability to produce abundant amounts of cellulolytic enzymes and are mainly exploited to produce cellulases (Sharada et al. 2013). However, cellulases from bacteria have been paid more attention because it has fast growth speed, wide adaption to extreme environment, and can produce more complex and muti-function cellulases to meet the industrial needs (Sadhu and Maiti 2013). Although numerous cellulases from different bacteria species have been studied and characterized (Rabinovich et al. 2002; Sadhu and Maiti 2013; Sharada et al.

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2013), the cellulases with novel properties are still needed to be further explored for bioenergy production.

Aqueous two-phase systems (ATPS) are formed by mixing certain amounts of polymers, salts, and water together, which have been extensively used to separate and purify nuclear acids, cells and organelles, enzymes, and proteins (Asenjo and Andrews 2011; Raja et al. 2012). ATPS have many advantages such as low toxic to environment, continuous to operate, and easy to scale up (Hatti-kaul 2001). It is one of low cost and high-efficient purification methods (Asenjo and Andrews 2011). Recently, many enzymes (α-amylase, α-galactosidase, lipase, proteinase), recombinant proteins, and antibody have been purified using this method (Azevedo et al. 2009; Porfiri et al. 2011; Gu et al. 2012; Loc et al. 2013; Ramakrishnan et al. 2016).

*Bacillus velezensis* A4 was isolated from forest soil previously in our lab, which exhibited strong ability in hydrolysis of lignocellulosic biomass (Guo et al. 2017). In order to further investigate the lignocellulosic enzyme properties from the A4 strain, the endoglucanase encoding gene was cloned and expressed in *Escherichia coli*. The enzyme was purified with ATPS and enzyme properties were characterized.

## Methods

### Bacterial strain and cultural condition

The *Bacillus* sp. A4 strain was used in this study which was isolated from the forest soil (Guo et al. 2017). The strain was activated by streaking in Luria–Bertani (LB) plate incubating at 37 °C overnight. Then a single colony was inoculated to 20 ml of liquid medium and cultured overnight in a shaker at 37 °C with 200 rpm. One percent (v/v) of the cultural broth was transferred into 50 ml mineral salt medium (MSM) containing 0.05% (w/v) carboxymethylcellulose sodium (CMC) and cultured in a shaker at 37 °C with 200 rpm for 80 h. The MSM medium consisted of (g/l) $KH_2PO_4$ 1.0 g, KCl 1.0 g, $NaNO_3$ 1.0 g, $MnSO_4$ 0.5 g, yeast extract 0.5 g, peptone 3 g, pH 7.0. The cultural medium was sterilized at 121 °C for 20 min before use.

### *rpoB* gene amplification and strain identification

The genome DNA was extracted using the method (Aljanabi 1997). The *rpoB* gene fragment was amplified with the primers CM7: 5′-AACCAGTTCCGCGTTGGCCTGG-3 (1383 bp) and CM31b: 5′-CCTGAACAACACGCTCGGA-3 (2473 bp) (Mollet et al. 1997). The PCR-amplified fragment was purified with DNA purification kit (Thermo Fisher Scientific, Canada) and sent for sequencing. The *rpoB* gene sequence of the strain was blasted with GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The highly similar *rpoB* gene sequence from different *Bacillus* strains was retrieved from GenBank and aligned using software Clustal X 1.83. The phylogenetic tree was constructed with software MEGA 7.01 using the neighbor-joining method.

### Egls gene cloning and sequence analysis

The endoglucanase gene (*egls*) fragment was amplified with the forward primer (5′-GGATCCATGCGAAGGAGGAAAAGATCAGAT) and reverse primer (5′-AAGCTTATTKGGTTCYGTTCCCCAAATCAGT). The restriction sites *Bam*HI and *Hind*III were shown underline. The PCR product was purified with a DNA purification kit (Thermo Fisher Scientific, Canada). The fragment was ligated to the vector pJET1.2-T and transformed into *E. coli* JM109. The plasmid was extracted and digested with enzymes *Bam*HI and *Han*dIII. The *egls* fragment was purified with Gel Extraction kit and ligated with the vector pET-21a in the corresponding sites. The degenerated plasmid was named pET21-egls, which has a 6 His-tag at the C-terminus of *egls* gene. The plasmid was sent to sequence and analyze.

The Nucleotide and amino acid sequences were analyzed with the online software BLASTn and BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved domains were predicted using bioinformatics tools (http://www.ncbi.nig.gov/structure/cdd/wrpsb.cgi). The signal peptide of Egls was predicted using online program Signal P 4.2 (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight (MW) and theoretical isoelectric point (PI) were analyzed using the software Vector NTI Advance 11.5.1. Multiple sequence alignment analysis was performed using software CLUSTAL × 1.8. Homology model was built using the online software (https://swissmodel.expasy.org/).

### Recombinant protein expression

The recombinant strain *E. coli* DE3 harboring the plasmid pET21-egls was inoculated into LB broth supplemented with ampicillin 50 mg/l. The strain was grown at 37 °C with shaking at 200 rpm overnight, and then it was inoculated to the same medium and was grown to OD600 0.5–0.6. After that, the recombinant strain was induced with 1 mM IPTG at 20 °C for 20 h. The cell pellets were collected in 50-ml conical tubes by centrifuging at room temperature for 10 min at 5000 rpm. The enzyme was purified with HisPur™ Ni–NTA Spin Purification Kit (Thermo fisher, Canada) under the native conditions. The recombinant protein was eluted with 500 mM imidazole in phosphate buffer (pH 8.0) and desalted with PD-10 column (Thermo fisher, Canada). The protein concentration was assayed according to the instruction of Bradford Protein Assay Kit (Bio Basic Canada Inc., Markham, Canada). SDS-PAGE was performed with 5% (w/v) stacking

gel and 12% (w/v) separating gel, respectively, under denature conditions. Zymogram analysis was carried out using the method (Lin et al. 2015).

### Enzyme activity assay

Endoglucanase activity was assayed according to the method (Ghose 1987) with minor modification using CMC as substrate. The reaction was carried out by adding 50 µl enzyme extraction into 50 µl of 0.05% (w/v) CMC substrate in 50 mM citrate buffer (pH 4.8) and incubated at 55 °C for 30 min. The released reducing sugars were measured by DNS method (Miller 1959), using glucose as standard curve. One unit of enzyme activity was defined as the amount of enzyme which could release 1 µg of reducing sugar in 1 min under assay conditions.

Exoglucanase activity and filter paper activity (FPA) assay was carried out in the same conditions as endoglucanase determination using 1% (w/v) Avicel and Whiteman No. 1 filter paper (0.3 × 0.5 cm) as substrate, respectively. The released reducing sugars from Avicel were determined by phenol–sulfuric acid method (Masuko et al. 2005), using glucose as standard curve. One unit of enzyme activity was defined as the amount of enzyme which could release 1 µg of reducing sugars in 1 min under assay conditions.

The β-glucosidase activity was assayed according to the method (Zhang et al. 2009), with minor modifications using pNPG as substrate. Briefly, 10 µl of enzyme was added into 140 µl of 1 mM pNPG substrate in 50 mM sodium citrate buffer (pH 4.8) and incubated at 55 °C for 30 min. Then 200 µl of 0.4 M glycine buffer (pH 10.8) was added into the reaction mixture. The appearance of yellow color was monitored at 430 nm by Microplate Spectrophotometer (Epoch, Bio Tek Instruments, Inc., Winooski, VT, USA). One unit of enzyme activity was defined as the enzyme released 1 mM of *p*-nitrophenyl per minute under assay conditions.

### Enzyme purification with ATPS

#### *Binodal curves preparation*

The bimodal curves were determined with turbidity method without NaCl (Asenjo and Andrews 2012). One g of 50% (w/w) PEG solution was added into a 15-ml tube. Then, the 40% (w/w) of citrate solution was added drop by drop, until the mixture becomes cloudy. The weight of PEG 4000 solution, phosphate buffer (pH 6.0), and the total weight of the tube were written down to calculate the ratio of PEG solution and citrate buffer solution in the system. After that some distilled water was added to make the system clear until one-phase system appeared. The above process was repeated 8–10 times until enough data were obtained to plot binodal curves.

#### *Enzyme purification with two-phase system*

Aqueous two-phase systems were prepared at room temperature by mixing certain amounts of 50% (w/w) of PEG solution, 40% (w/w) of phosphate buffer, 2 ml cell culture supernatant sonicated in 50 mM of citrated buffer (pH 6.0), and NaCl in 15 ml centrifuge tubes with conical cap. pH was pre-adjusted to (6.0–8.0) in phosphate buffer. Distilled water was added to the system to obtain 10 g of total weight. After vortex for 5 min, the tube was centrifuged at 5000 rpm for 3 min to make the phase separate. Different concentrations of PEG 4000 (10–25% w/w), phosphate buffer (8–18% w/w), buffer pH (6–8), and NaCl (0–15%, w/w) were used to study the partition of the recombinant enzyme in ATPS.

Partition coefficient ($K$) is the ratio of protein partitioned in the top phase to that of in the bottom phase. Purification factor (PF) is defined as the ratio of specific activity of the phase enzyme participated to that of the crude enzyme. Enzyme recovery (ER) is defined as the ratio of the total enzyme recovered from the phase to the total enzyme activity in crude enzyme.

### Biochemical characterizations of Egls

#### *The optimal pH and temperature*

The optimal pH and temperature for endoglucanase activities were measured in different pH values (3.0–10.0) and various temperatures (30–75 °C), respectively, using 0.05% (w/v) of CMC in citrate buffer (pH 4.8) as substrates. Sodium acetate buffers (50 mM) were used to maintain the pH range of 3.0–5.0, phosphate buffer (50 mM) 6.0–8.0, and glycine–NaOH buffer (50 mM) 9.0–10.0, respectively. The enzyme activity compared to the highest activity in percentage (%) in the reaction conditions was shown as relative activity.

#### *The thermal and pH stability*

In thermal and pH stability assay, the purified Egls (50 µg/ml) was incubated at temperature range of 30–80 °C for 15–60 min in 50 mM citrate buffer (pH 4.8) at pH 3.0–10.0 for 5 h at room temperature. The remained enzyme activity was measured under standard conditions. The original enzyme activity was set as 100%. The relative enzyme activity (%) was defined as the remained enzyme activity compared with original enzyme activity.

#### *Effects of metal ions and chemicals on endoglucanase on enzyme activity*

Effects of metal ions and chemicals on Egls activities were determined using 2 and 10 mM of different metal irons ($CaCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$, $ZnCl_2$, $CuCl_2$, and KCl) in 50 mM citrate buffer (pH 4.8) and chemicals (SDS, Triton-X 100, Tween-20, EDTA and PMSF), respectively.

The enzyme activities were assayed under standard conditions. The enzyme activity without addition of metal ions was defined as 100% and used as a control. The results were shown as relative activity (%).

#### *Kinetic parameters $K_m/k_{cat}$ assay*

Kinetic parameters $K_m$, $V_{max}$, and $k_{cat}$ were determined according to the procedure (Allison 2001). Twenty-two U/ml of enzyme was used to digest 1–10 mg/ml of CMC substrate under optimized conditions. The data were analyzed and the Lineweaver–Burk plot was made by the software Origin version 8.0.

#### *Substrate specificity assay*

Different substrates were used to evaluate the specificities of the purified enzyme Egls under the optimized conditions. The enzyme activity was measured under standard condition. The glucose was used as standard.

#### *Statistic analysis*

All the experiments were carried out in triplicate. The results were shown as mean ± standard deviation. One-way analysis of variance (ANOVA) was applied to analyze the significance of data. A $p < 0.05$ was considered as statistically significant in data using Tukey's HSD method.

#### *Accession numbers*

The gene sequence of *egls* and *rpoB* were submitted to the GenBank with the Accession No (MG748603 and MG748604), respectively.

## Results and discussion

### *B. velezensis* A4 growth and enzyme production

The strain was inoculated at solid MSM medium with CMC (0.05% w/v) for 48 h. A big halo in petri dish indicated that the bacteria could produce a large amount of cellulase to hydrolyze the substrate (Fig. 1a). The strain was grown in MSM medium at 37 °C for 80 h, and the cell growth and enzyme production were determined (Fig. 1b). The bacteria grew very quickly at first 8 h and the cell density reached the highest amount at 16 h. The cell density almost remained constant over the times from 16 to 64 h. The enzyme production began at 16 h and reached the highest amount (21.71 U/ml) at 48 h.

### Strain identification

This strain was identified as *Bacillus* sp. A4 previously by 16S rDNA (Guo et al. 2017), which showed 99% of identity with strain *B. velezensis*, *Bacillus amyloliquefaciens*, and *Bacillus* sp. These strains are very close in evolutionary relationship. The genus *Bacillus* is one of the most important enzyme production microorganisms, which consisted of 318 species (Dworkin et al 2006). Some *Bacillus* species such as *B. velezensis*, *B. amyloliquefaciens*, and *Bacillus siamensis* are highly closed in genetic relationship and it is hard to identify the species by 16S rDNA methods (Fan et al. 2017). *rpoB* gene was found more useful to identify the strain within species than 16S rDNA (Mollet et al. 1997). The *rpoB* gene is more sensitive in identifying the closest strains in evolutionary relationship, which has been used to identify the species

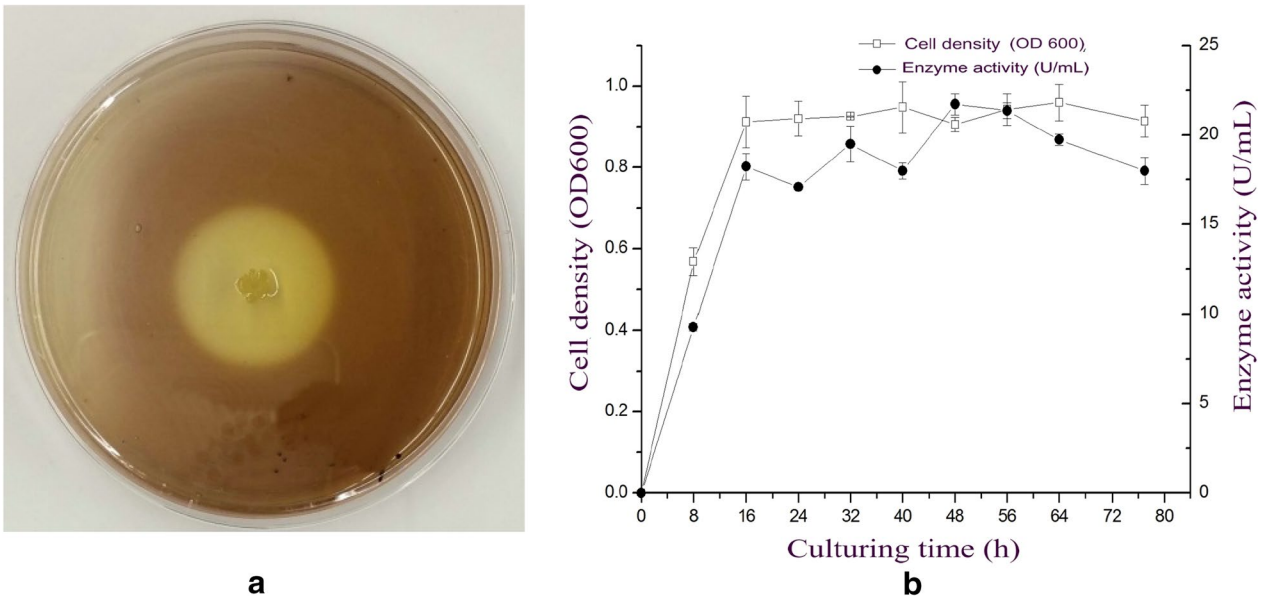


**Fig. 1** **a** The halo of the strain *B. velezensis* A4 on CMC plate. The strain was grew on MSM + CMC (0.1% w/v) medium at 37 °C for 48 h and stained with 1% (w/v) iodine solution; **b** the growth curve of *B. velezensis* A4. The strain was inoculated in 50 ml MSM liquid medium in a 250-ml Erlenmeyer flask and cultured at 37 °C for 80 h in a shaker at 200 rpm

(Mollet et al. 1997; Drancourt and Raoult 2002; Ki et al. 2009). The *rpoB* gene of strain *Bacillus* sp. A4 was amplified with primers, sequenced, and homology blast performed with related species in GenBank. Results showed that the strain has 100% similarity with *B. velezensis* (Fig. 2). So we propose this strain belongs to *B. velezensis.* We named it *B. velezensis* A4.

### Egls gene sequence analysis

The gene *egls* (GenBank Accession No. MG748603) has an open reading frame of 1521 bp, which encodes a protein of 507 amino acids with a calculated molecular weight 55.7 kD and isoelectric point at 9.11. The enzyme protein has a signal peptide at N-terminal (1–37 residues), predicted by Signal P 4.2 web software (Fig. 3).

The gene sequence *egls* showed 99% of sequence identity with the stain *B. velezensis* JS25R (GenBank Accession No. CP009679.1). Multiple amino acid BLAST analysis revealed that the enzyme Egls (residues 6–499) showed 100% sequence identity with glycoside hydrolase family 5 from *B. velezensis* (WP_022553273.1), but the first 5 amino acid is different (Fig. 4a).

Computer model of enzyme Egls shows that it consists of a $(\alpha/\beta)_8$ barrel, 7 $\beta$ sheet strands, and a Manganese(II) binding site (Fig. 4b). The proposed conserved domains have cellulase domain (residues 50–296) (glycosyl hydrolase family 5), a CBM_3 cellulose-binding domain (residues 356–437), and one $Mg^{2+}$ binding site, related with amino acids (G157, D195, D197, N198). The enzyme showed 96.73% sequence identity with endoglucanase. Some strains of *B. velezensis* species have been sequenced and endoglucanases were annotated in GenBank by bioinformatics methods. However, the enzyme characterizations were not been clearly investigated.

### Expression and purification of the recombinant enzyme

To investigate the enzyme function of Egls, the encoding sequence of egls with signal peptide sequence from *B. velezensis* A4 was cloned and expressed in *E. coli* (Fig. 5a). The enzyme was expressed using pET-21a vector, which has a C-terminal His-tag. The recombinant enzyme was induced and expressed with different concentrations (0.2–1.0 mM) of IPTG. The enzyme showed the highest CMCase activity (28.06 ± 1.04 U/ml) and filter activity (16.07 ± 0.87 U/ml) induced with 0.4 mM of IPTG, but no activity was detected in the cell pellet and supernatant of the culture broth. The recombinant enzyme was purified to homogeneity using NTA affinity column (data not shown). The molecular mass of Egls is shown 55 kD in zymogram (Fig. 5b). Similarly, the endoglucanase from *Bacillus subtilis* was expressed and purified with molecular weight of 55 kD (Li et al. 2008). However, no enzyme activity was detected when purified with NTA affinity column. This may be because His-tags generally have some minor effect on the structure of the native enzyme (Carson et al. 2007). His-tags are known to mediate oligomerization via metal cation and can interact with some metal ions ($Ca^{2+,}$ $Mg^{2+}$), which may affect the enzyme activity.

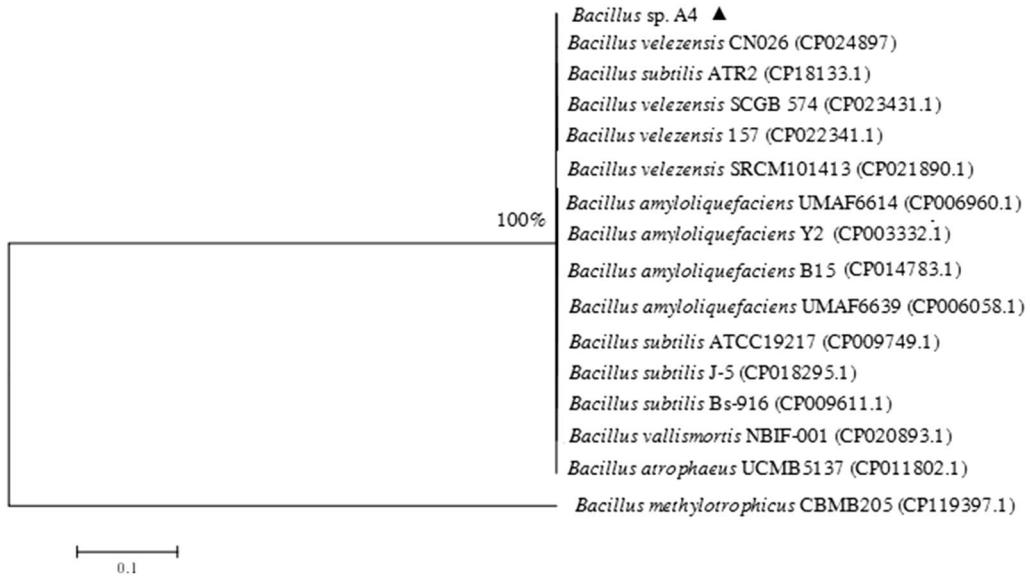


**Fig. 2** Phylogenetic tree was constructed by neighbor-joining method using *rpoB* gene sequence obtained from different *Bacillus* strains in GenBank. The numbers at the branches indicate the confidence level calculated by bootstrap analysis (1000). The scale bar shows the evolutionary distance between species. The numbers in the brackets are the accession numbers of *rpoB* gene sequences from GenBank

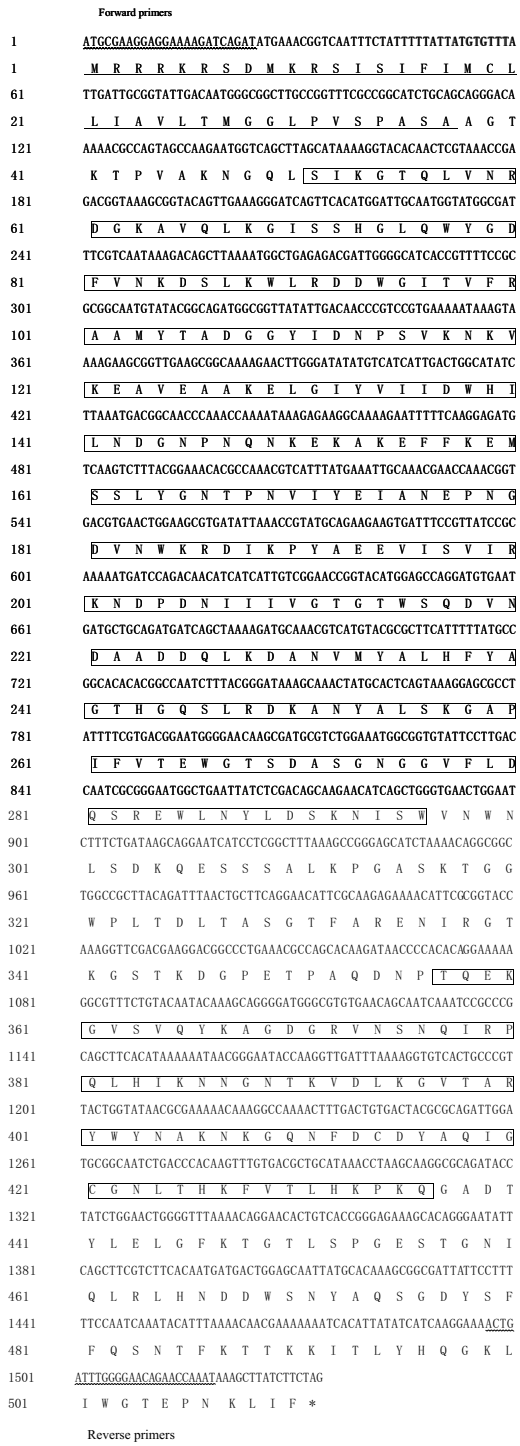


**Fig. 3** Nucleotide sequence and predicted amino acid sequence of Egls from *B. velezensis* A4. The underlined amino acid (1–37 amino acids) shows the putative signal peptide. The wave lines indicate the primers. The regions of amino acids (50–296) and (356–437) predicted as cellulose domain (glycosyl hydrolase family 5) and CBM_3 cellulose-binding domain, respectively, are depicted in boxes. Asterisk indicates the stop codon

Due to the enzyme activity loss in NTA affinity column, the recombinant enzyme Egls was re-purified using PEG 4000, phosphate buffer, and NaCl aqueous two-phase system. Aqueous two-phase system is one of the most economical, high-efficient, easy-to-operate, and scale-up methods, which has been extensively used in bio-products purification, such as enzymes, proteins, nucleic acids, and amino acids (Ratanapongleka 2010). The factors, such as polymer molecular weight, pH, temperature, biomolecule size, surface charge, also contribute to the partition behavior of biomolecules between the two phases (Ratanapongleka 2010). The Binodal curves were built to use as a guideline for the enzyme purification in ATPS (data not shown). Different concentrations of PEG4000, $K_2HPO_4$/$NaH_2PO_4$ buffer, NaCl, and buffer pH were exploited to find the optimized condition for enzyme purification (Fig. 6). Results showed that 25% (w/w) of PEG 4000, phosphate buffer 8.08% (w/w) (pH 6.0), and NaCl (5% w/w) have the highest enzyme recover rate (90.39%) and purification factor (12.05) (Table 1). These parameters were used to purify recombinant enzyme Egls from supernatant of the cells. The recombinant enzyme Egls was partitioned to the bottom phase (salt-rich phase) of this system. It has been found that some enzymes would tend to partition to the bottom phase when it was purified with two-phase system (Gu et al. 2012; Loc et al. 2013; Ramakrishnan et al. 2016). An increase of pH and NaCl concentration in the two-phase system leads to the decrease of recovery rate of Egls. The concentrations of polymers, buffers, and NaCl have great impact on the partition of protein in aqueous two-phase system (Asenjo and Andrews 2012).

## Biochemical properties of Egls

### *The optimal pH and temperature*

The effects of pH and temperature on crude recombinant enzyme and purified enzyme activities were analyzed at different pH values (3.0–10.0) and temperatures (30–80 °C) under standard conditions, respectively. Results showed that the enzyme has an optimal temperature at 55 °C and optimal pH at 5.0–6.0 and (Fig. 7a, b). The enzyme characteristics were mainly determined by the structure of Egls. The optimal temperature and pH of endoglucanase were different from the different sources of *Bacillus* species (Table 2).

### *The thermal and pH stability*

The thermal stability was assayed at different temperatures (55–70 °C) for 1 h. The results showed that the enzyme was inactivated after incubation at 70 °C for 30 min (Fig. 7c, d). The crude enzyme and the purified enzyme were stable at the pH range of 5.0–7.0. Egls was not thermostable at temperatures above 70 °C and it was

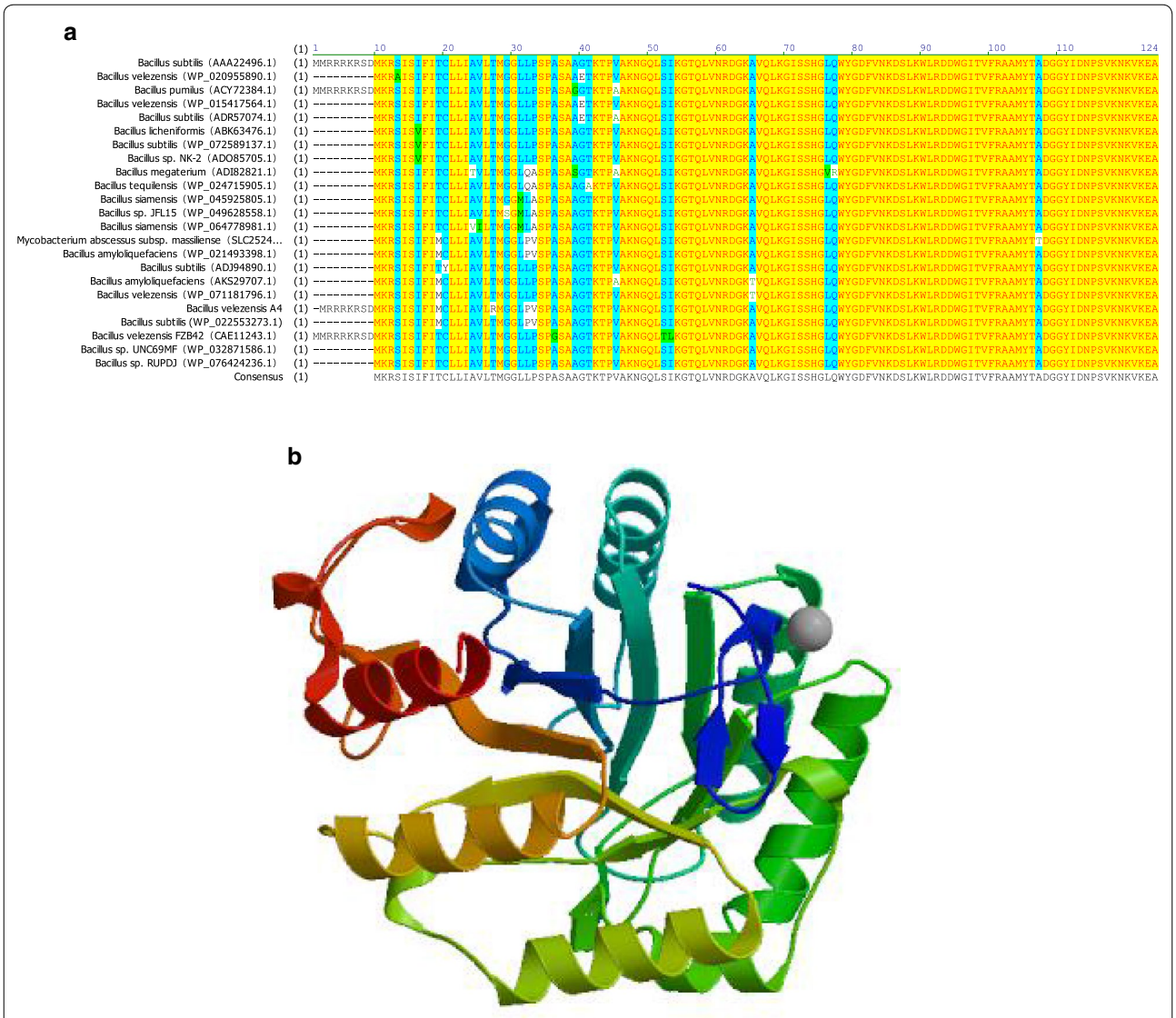


**Fig. 4** **a** Multiple sequence alignment of endoglucanase (Egls) amino acid sequence with endoglucanase from other *Bacillus* strains. Note: There are 5 different amino acids in the front of Egls from *B. velezensis* A4; **b** homology model of Egls using predicted amino acid as template, which consists of α-helixes, β-strands, random coils, $Mg^{2+}$ binding site (grew ball)

stable at nearly neutral pH environment. The enzyme properties are highly related to the structure of the enzyme. Some endoglucanases from *Bacillus* have higher thermostability, and others have a wider range of pH stability (Table 2).

### *Effects of metal ions and chemicals on endoglucanase on enzyme activity*

The effects of different metal ions and chemicals on enzyme activity were analyzed (Table 3). Two mM of $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, and $Co^{2+}$ slightly increased enzyme activity (5–10%), while $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and $Mn^{2+}$ almost have no effects on the enzyme activity. Ten mM of $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, and $Co^{2+}$ increased the enzyme activity (15–30%). Chemicals such as Triton-X 100, Tween-20 (5% w/v) increased the enzyme activity about 5 and 22%, respectively. 5% (w/v) EDTA slightly decreased the enzyme activity. However, PMSF strongly inhibited the enzyme activity.

### *Kinetic parameters $K_m/K_{cat}$*

The kinetic parameters $K_m/K_{cat}$ were determined using various concentrations of CMC as substrate and analyzed with Lineweaver–Burk plot. The $K_m$, $V_{max}$, and $K_{cat}$ values of Egls were exhibited as 63.38 mg/ml, 55.6 mg/min, and $3.93 \times 10^3$/S, respectively. The value $K_m/K_{cat}$ of Egls was

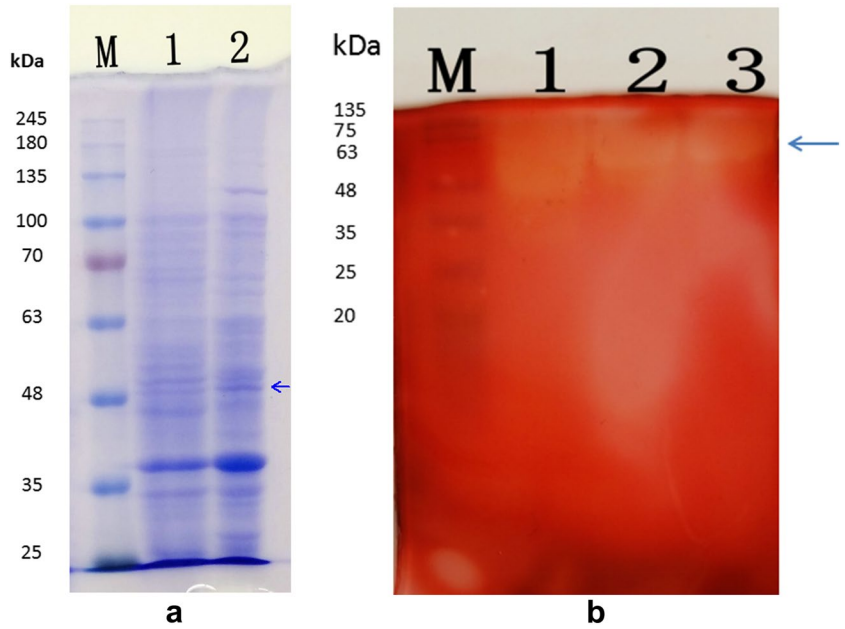


**Fig. 5** **a** SDS-PAGE pattern of endoglucanase from pET21-egls/DE3 purified with ATPS. M Protein ladder; 1 control (without IPTG induction); 2 pET-21-egls (induced with 1 mM IPTG); **b** zymogram analysis of crude enzyme and purified Egls. M Protein ladder; 1 crude enzyme (pET21-egls/DE3); 2 purified enzyme (pET 21-egls/DE3)

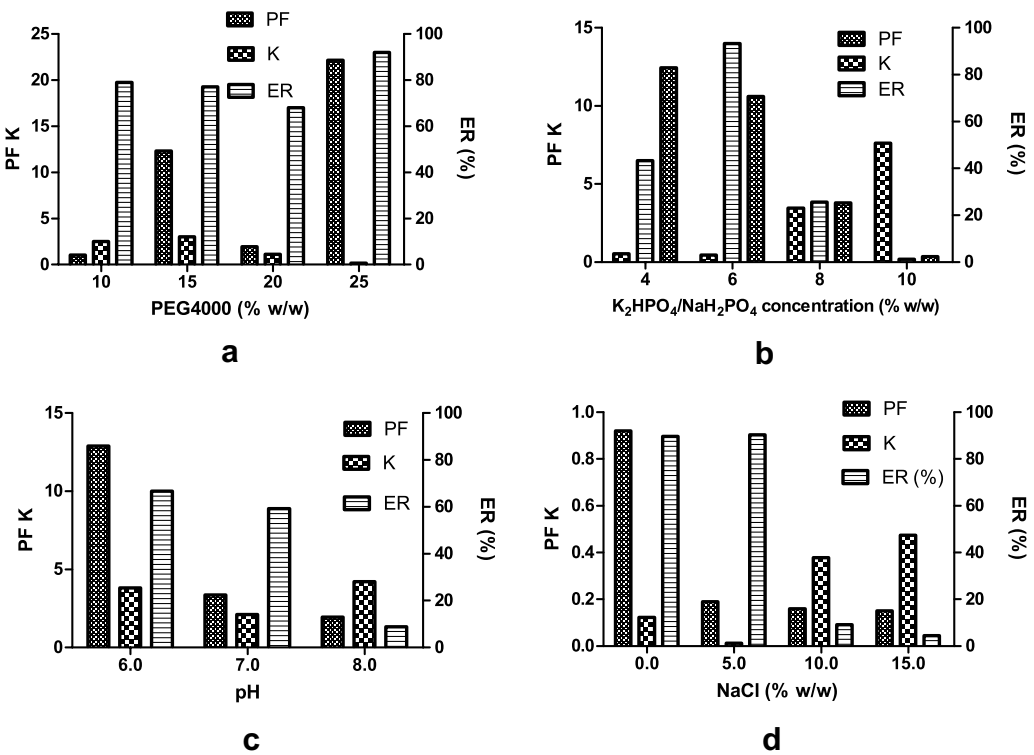


**Fig. 6** Effect of PEG 4000, NaCl concentration, and pH on purification of endoglucanase from supernatants of pET21-egls/DE3 cells. *PF* purification factors, *K* partition coefficient, *ER* enzyme recovery. **a** PEG 4000 (10–25%, w/w); **b** K2HPO4/NaH2PO4 (4–10%, w/w); c pH (6.0–8.0); d NaCl (0–15%, w/w)

**Table 1 Purification of recombinant enzyme Egls using PEG 4000-phosphate-NaCl system**

| Steps | Volume | Total protein (μg/ml) | Total CMCase (U/ml) | PF | CR | ER (%) |
|---|---|---|---|---|---|---|
| Crude enzyme | 40 | 22,330.44 | 1105.65 | 12.05 | 3.18 | 90.39 |
| Top phase | 140 | 2611.30 | ND | | | |
| Bottom phase | 44 | 3235.91 | 1223.25 | | | |

*ND* not detected

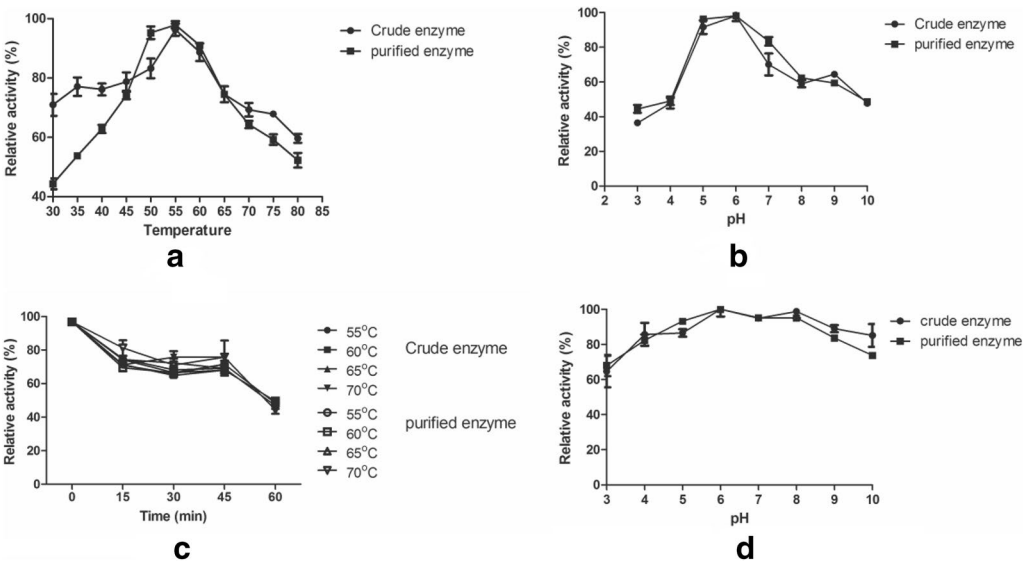


**Fig. 7** Effect of pH and temperature on enzyme activities of Egls: **a** optimal temperature; **b** optimal pH; **c** pH stability; **d** thermal stability

**Table 2 Endoglucanase properties from different *Bacillus* strains**

| *Bacillus* strains | Molecular weight (MW) (kDa) | Optimal pH | Optimal temperature (°C) | pH stability | Thermostability | Reference |
|---|---|---|---|---|---|---|
| *B. velezensis* A4 | 55 | 6.0 | 55 | 5.0–8.0 | 65 °C 30 min | This study |
| *B. subtilis* | 55 | | 50 | | 75 °C 30 min | Li et al. (2008) |
| *B. amyloliquefaciens* | 54 | 7.0 | 50 | | | Lee et al. (2008) |
| *B. licheniformis* | 37 | 9.0 | 50 | 12 | 80 °C | Annamalai et al. (2012) |
| *B. subtilis* JS2004 | 63 | 9.0 | 50 | | | Zafar et al. (2014) |

calculated as $1.74 \times 10^{-2}$ mg/ml S. The kinetic parameters ($K_m$, $V_{max}$, and $K_{cat}$) of an endoglucanase from *Bacillus* strain were found as 0.25 mg/ml, 20 μmol/ml/min, and 0.55/S respectively (Sadhu et al. 2013). The $K_m$ of endoglucanase from the strain *Hahella chejuensis* KCTC 2396 was 1.8 mg/ml (Ghatge et al. 2014). The higher $K_m$ value of our strain indicated that the enzyme Egls is less efficient in digesting CMC substrate than reported strains.

### *Substrate specificity*

The substrates specificity was studied using different substrates (Table 4). Results showed that the purified enzyme Egls has higher CMC activity (75.61 ± 8.45 U/ml) and FPA activity (69.22 ± 8.52 U/ml) than that of the crude enzyme, which indicated that the enzyme has endoglucanase activity. The purified enzyme has higher enzyme activity than crude enzyme, because the purified enzyme has more enzyme molecules than crude enzyme

**Table 3 Effect of metal irons and chemicals on purified recombinant enzyme Egls activities**

| Metal ions | Relative activity (%) | | Chemicals | Relative activity (%) | |
|---|---|---|---|---|---|
| | 2 MM | 10 MM | | 2% (w/v) | 5% (w/v) |
| Control | 100.3 ± 1.0 | 100.3 ± 1.0 | Control | 100.3 ± 1.0 | 100.3 ± 1.0 |
| $Ca^{2+}$ | 105.2 ± 2.3 | 115.7 ± 5.2[a] | Triton-X 100 | 105.0 ± 4.4 | 122.9 ± 5.8[a] |
| $K^{+}$ | 105.5 ± 1.8 | 104.3 ± 4.6 | Twen-20 | 100.8 ± 3.6 | 105.2 ± 2.5 |
| $Mg^{2+}$ | 111.0 ± 1.4[a] | 134.8 ± 11.1[a] | EDTA | 99.6 ± 0.6 | 96.7 ± 0.5 |
| $Mn^{2+}$ | 101.7 ± 1.7 | 134.2 ± 4.9[a] | PMSF | ND | ND |
| $Ni^{2+}$ | 98.8 ± 3.1 | 98.6 ± 3.6 | | | |
| $Zn^{2+}$ | 100.3 ± 3.4 | 122.0 ± 1.8[a] | | | |
| $Cu^{2+}$ | 100.0 ± 3.0 | 103.5 ± 4.8 | | | |
| $Co^{2+}$ | 110.1 ± 1.1[a] | 116.5 ± 3.8[a] | | | |

*ND* not detected

[a] Significant difference ($p < 0.05$)

**Table 4 Substrates specificity of crude and purified recombinant enzyme Egls**

| Substrates | Crude enzyme (U/ml) | Purified enzyme (U/ml) |
|---|---|---|
| CMC | 22.06 ± 4.61 | 75.61 ± 8.45* |
| Filter | 40.84 ± 6.38 | 69.22 ± 8.52* |
| Avicel | 63.86 ± 16.10 | 68.84 ± 24.81* |
| pNPG | ND | ND |

*ND* not detected

* Significant difference ($p < 0.05$)

in equal reaction volume and PEG 4000 existed in purified enzyme makes the enzyme more stable and increases enzyme activity. The purified enzyme Egls has an enzyme activity (68.84 ± 24.81 U/ml) using Avicel as substrates, which was also higher than that of crude enzyme (63.86 ± 16.10 U/ml). It indicated that the enzyme has both endoglucanase activity and exoglucanase activity. The enzyme cannot digest pNPG, which indicates that Egls has no β-glucanase activity. This result was in agreement with the findings that some cellulases from *Bacillus* showed both endoglucanase activity and exoglucanase activity (Sadhu et al. 2013; Ghatge et al. 2014; Wei et al. 2015). Egls is very useful for the pretreatment of lignocellulose of biomass to produce reducing sugar that is used in bio-products and bioenergy production.

## Conclusion

We have cloned and expressed *egls* gene from *B. velezensis* A4 in *E. coli* BL21 (DE3). The recombinant enzyme was purified with PEG 4000, phosphate, and NaCl aqueous two-phase system. Although gene sequence was highly similar to other *B. velezensis* stain JS25R, this study is the first report of the recombinant expression, purification, and characterization of the enzyme. The recombinant enzyme is an endoglucanase, has an optimal temperature at 55 °C and pH range of 5.0–6.0, is stable at pH range of 5.0–7.0, and is actively supplemented with a series mental ions and can be used in biomass pretreatment to produce reducing sugars in bioenergy production. The study of the enzyme properties of the predicted cellulase genes is very important for the enzyme application in biomass degradation and bioenergy production.

#### Authors' contributions

YL conceived, designed, and performed the experiments. HG constructed the *E. coli* expression vector. YW performed a part of the experiments. WQ was involved in project planning, experimental designing, manuscript revisions, and editing. All authors read and approved the final manuscript.

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#### Competing interests

The authors declare that they have no competing interests.

#### Availability of data and materials

All data obtained or analyzed in this work are included in this article.

#### Consent for publication

Not applicable.

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Not applicable.

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### References


Aljanabi S (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res 25:4692–4693. https://doi.org/10.1093/nar/25.22.4692

Allison RD (2001) Kinetic assay methods. Curr Protoc Mol Biol Appendix 3

Annamalai N, Rajeswari MV, Elayaraja S et al (2012) Purification and characterization of thermostable alkaline cellulase from marine bacterium *Bacillus licheniformis* Au01 by utilizing cellulosic wastes. Waste Biomass Valoriz 3:305–310. https://doi.org/10.1007/s12649-012-9113-y

Asenjo JA, Andrews BA (2011) Aqueous two-phase systems for protein separation: a perspective. J Chromatogr A 1218:8826–8835. https://doi.org/10.1016/j.chroma.2011.06.051

Asenjo JA, Andrews BA (2012) Aqueous two-phase systems for protein separation: phase separation and applications. J Chromatogr A 1238:1–10. https://doi.org/10.1016/j.chroma.2012.03.049

Azevedo AM, Gomes AG, Rosa PAJ et al (2009) Partitioning of human antibodies in polyethylene glycol-sodium citrate aqueous two-phase systems. Sep Purif Technol 65:14–21. https://doi.org/10.1016/j.seppur.2007.12.010

Basu P (2013) Biomass gasification, pyrolysis and torrefaction: practical design and theory. Academic Press, Boston. https://doi.org/10.1016/C2011-0-07564-6

Carson M, Johnson DH, McDonald H et al (2007) His-tag impact on structure. Acta Crystallogr Sect D: Biol Crystallogr 63:295–301. https://doi.org/10.1107/S0907444906052024

Drancourt M, Raoult D (2002) *rpo*B Gene sequence-based identification of *Staphylococcus* species. J Clin Microbiol 40:1333–1338. https://doi.org/10.1128/JCM.40.4.1333

Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) (2006) The prokaryotes. A handbook on the biology of bacteria. Springer, New York. https://doi.org/10.1007/0-387-30744-3

Fan B, Blom J, Klenk HP, Borriss R (2017) *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "Operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. Front Microbiol 8:22. https://doi.org/10.3389/fmicb.2017.00022

Garvey M, Klose H, Fischer R et al (2013) Cellulases for biomass degradation: comparing recombinant cellulase expression platforms. Trends Biotechnol 31:581–593

Ghatge SS, Telke AA, Kang SH et al (2014) Characterization of modular bifunctional processive endoglucanase Cel5 from *Hahella chejuensis* KCTC 2396. Appl Microbiol Biotechnol 98:4421–4435. https://doi.org/10.1007/s00253-013-5446-0

Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268. https://doi.org/10.1351/pac198759020257

Gu F, Gao J, Xiao J et al (2012) Efficient methods of purification of α-galactosidase from *Aspergillus niger*: aqueous two-phase system versus three-phase partitioning. Rom Biotechnol Lett 17:7853–7862

Guo H, Lin C, Wang S et al (2017) Characterization of a novel laccase-producing *Bacillus* sp. A4 and its application in *Miscanthus* degradation. BioResources 12:4776–4794

Hatti-kaul R (2001) Aqueous two-phase systems. Mol Biotechnol 19:697–713. https://doi.org/10.1385/MB:19:3:269

Ki JS, Zhang W, Qian PY (2009) Discovery of marine *Bacillus* species by 16S rRNA and *rpo*B comparisons and their usefulness for species identification. J Microbiol Methods 77:48–57. https://doi.org/10.1016/j.mimet.2009.01.003

Lee YJ, Kim BK, Lee BH et al (2008) Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. Bioresour Technol 99:378–386. https://doi.org/10.1016/j.biortech.2006.12.013

Li W, Zhang WW, Yang MM, Chen YL (2008) Cloning of the thermostable cellulase gene from newly isolated *Bacillus subtilis* and its expression in *Escherichia coli*. Mol Biotechnol 40:195–201. https://doi.org/10.1007/s12033-008-9079-y

Lin C, Shen Z, Zhu T, Qin W (2015) Newly isolated *Penicillium ramulosum* N1 is excellent for producing protease-resistant acidophilic xylanase. J Mol Microbiol Biotechnol 25:320–326. https://doi.org/10.1159/000439170

Loc NH, Thi H, Lien T et al (2013) Purification of recombinant neutral protease (NPRC10) by partitioning in aqueous two-phase systems. Eur J Exp Biol 3:252–257

Masuko T, Minami A, Iwasaki N et al (2005) Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339:69–72. https://doi.org/10.1016/j.ab.2004.12.001

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428. https://doi.org/10.1021/ac60147a030

Mollet C, Drancourt M, Raoult D (1997) *rpoB* sequence analysis as a novel basis for bacterial identification. Mol Microbiol 26:1005–1011. https://doi.org/10.1046/j.1365-2958.1997.6382009.x

Pérez J, Muñoz-Dorado J, De La Rubia T, Martínez J (2002) Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int Microbiol 5:53–63

Porfiri MC, Picó G, Romanini D, Farruggia B (2011) *Aspergillus oryzae* alpha-amylase partition in potassium phosphate-polyethylene glycol aqueous two-phase systems. Int J Biol Macromol 49:7–13. https://doi.org/10.1016/j.ijbiomac.2011.03.003

Rabinovich ML, Melnik MS, Bolobova AV (2002) Microbial cellulases (review). Appl Biochem Microbiol 38:305–321

Raja S, Murty VR, Thivaharan V et al (2012) Aqueous two phase systems for the recovery of biomolecules—a review. Sci Technol 1:7–16. https://doi.org/10.5923/j.scit.20110101.02

Ramakrishnan V, Goveas LC, Suralikerimath N et al (2016) Extraction and purification of lipase from *Enterococcus faecium* MTCC5695 by PEG/phosphate aqueous-two phase system (ATPS) and its biochemical characterization. Biocatal Agric Biotechnol 6:19–27. https://doi.org/10.1016/j.bcab.2016.02.005

Ratanapongleka K (2010) Recovery of biological products in aqueous two phase systems. Int J Chem Eng Appl 1:191–198. https://doi.org/10.7763/IJCEA.2010.V1.33

Sadhu S, Maiti TK (2013) Cellulase production by bacteria: a review. Br Microbiol Res J 3:235–258. https://doi.org/10.9734/BMRJ/2013/2367

Sadhu S, Saha P, Sen SK et al (2013) Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. Springerplus 2:1–10. https://doi.org/10.1186/2193-1801-2-10

Sharada R, Venkateswarlu G, Venkateshwar S, Rao MA (2013) Production of cellulase—a review. Ijpcbs 3:1070–1090

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review q. Bioresour Technol 83:1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

Wei KSC, Teoh TC, Koshy P et al (2015) Cloning, expression and characterization of the endoglucanase gene from *Bacillus subtilis* UMC7 isolated from the gut of the indigenous termite *Macrotermes malaccensis* in *Escherichia coli*. Electron J Biotechnol 18:103–109. https://doi.org/10.1016/j.ejbt.2014.12.007

Yang TC, Kumaran J, Amartey S et al (2014) Biofuels and bioproducts produced through microbial conversion of biomass. Bioenergy Res Adv Appl. https://doi.org/10.1016/B978-0-444-59561-4.00005-X

Zafar M, Ahmed S, Khan MIM, Jamil A (2014) Recombinant expression and characterization of a novel endoglucanase from *Bacillus subtilis* in *Escherichia coli*. Mol Biol Rep 41:3295–3302. https://doi.org/10.1007/s11033-014-3192-8

Zhang YHP, Hong J, Ye X (2009) Cellulase assay. Biofuels Methods Protoc Methods Mol Biol 581:213–231. https://doi.org/10.1007/978-1-60761-214-8