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Research Article

On the expression of recombinant Cas9 protein in *E. coli* BL21(DE3) and BL21(DE3) Rosetta strains

Gabriela Pannunzio Carmignotto, Adriano Rodrigues Azzoni*

Departamento de Engenharia Química, Escola Politécnica, Universidade de São Paulo, CEP 05508-010, São Paulo, SP, Brazil

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ABSTRACT

The CRISPR-Cas9 system is a new tool that has been extensively used for genome editing. The system is composed of a Cas9 endonuclease, which has the function of cleaving DNA at a specific site, and a guide RNA (gRNA), which contains the sequence of the cleavage site that is the target of editing. Despite the great interest that has been generated because of the utility of Cas 9 as a molecular tool and a potential therapeutic protein, the production of the 158 kDa recombinant Cas9 protein derived from *Streptococcus pyogenes* remains a challenge. Here, we systematically evaluated the expression of recombinant Cas9 protein in two different *E. coli* strains in complex and defined media. The recombinant protein showed improved expression in *E. coli* BL21(DE3), while only traces of Cas9 protein could be detected in the Rosetta (DE3) strain as a result of much lower mRNA levels. The greatest Cas9 protein expression in defined media containing glucose was observed at an induction temperature of 30 °C and with 8 h of post induction time using IPTG in shake flasks. The protein concentration obtained during a batch bioreactor culture was approximately 420.1 mg/L with 6 h of post induction time. The results demonstrated the possibility of efficient Cas9 protein expression in batch mode using *E. coli* BL21(DE3) and a simple defined medium and also showed the potential for further improvements that could facilitate large-scale production.

1. Introduction

The clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) system is a powerful tool that has been developed and used for genome editing (Jinek et al., 2012; Doudna and Charpentier, 2014; Liu et al., 2017). The CRISPR-Cas9 system is able to modify DNA sequences with high specificity and efficiency by means of a complex composed of the endonuclease Cas9 and a single guide RNA (sgRNA) (Mali et al., 2013; Cho et al., 2013). The Cas9 protein has the function of cleaving the DNA, while the sgRNA is responsible for directing the Cas9 protein to a specific DNA sequence (Jinek et al., 2012). This new technology has been used *in vitro* at the cellular level but also has potential for use in *in vivo* therapeutic applications (Gagnon et al., 2014; Ran et al., 2015; Dong et al., 2015; Yang et al., 2017; Chira et al., 2017; Kawabe et al., 2018). In 2016, a research group from China began the first clinical trial that used CRISPR-Cas9 technology to edit *ex vivo* immune cells from a patient with lung cancer (Cyranoski, 2016).

To ensure the success of the therapeutic application of genome editing, the Cas9/sgRNA system has to be delivered to the target cells (Glass et al., 2018). The CRISPR-Cas9 system components can be delivered in three different forms: 1) delivery of the plasmid encoding the Cas9 protein and the sgRNA, 2) delivery of Cas9 mRNA and sgRNA, and 3) delivery of Cas9 protein and sgRNA (Ran et al., 2015; Chu et al., 2016; Kim et al., 2016). All three delivery strategies have advantages and disadvantages. The main advantage of using the plasmid-based system is that it allows the delivery of one stable molecule (Maddalo et al., 2014; Li et al., 2018). However, gene delivery introduces the risk of plasmid integration into the host genome (Kim et al., 2016; Cradick et al., 2013; Timim et al., 2018). Furthermore, the introduction of plasmids allows for an increase in the amount of time of protein and sgRNA expression, which can increase off-target effects and immunogenic responses (Gaj et al., 2012; Liang et al., 2015). Another option is the delivery of Cas9 mRNA and sgRNA (Timim et al., 2018). Although the use of mRNA allows transient expression, which can decrease off-target effects, mRNA is less stable than DNA, and the results obtained so far indicate that genome editing using this form of CRISPR-Cas9 system delivery produces low efficiency (Chu et al., 2016; Li et al., 2018). The third alternative utilizes the delivery of the Cas9 nuclease and the sgRNA complex. Despite the difficulties in efficient Cas9-sgRNA delivery resulting from the large size of the complex, this delivery technique has shown promising results (Liang et al., 2015; Ramakrishna et al., 2014; Kim et al., 2018; Wu et al., 2018). This method provides better control of the intracellular concentration of the CRISPR-Cas9 system, more rapid activity, decreased immunogenic responses and

* Corresponding author.
E-mail address: adriano.azzoni@usp.br (A.R. Azzoni).

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reduced off-target effects (Sun et al., 2015; Liang et al., 2015; Zuris et al., 2015; Kim et al., 2016; DeWitt et al., 2017). Different methods have been used to deliver CRISPR-Cas9 components, such as electroporation, microinjection, lipid and polymer nanoparticles, cell-penetrating peptides, and adeno-associated viruses (Ran et al., 2015; Ramakrishna et al., 2014; Timim et al., 2018; Li et al., 2018).

Due to the advantages and promising results of the use of CRISPR-Cas9 in genome editing studies, the recombinant Cas9 protein has been increasingly produced at laboratory scale. Surprisingly, few studies presented in the literature have minimally focused on the production of the Cas9 protein at a small scale, in laboratories (Ramakrishna et al., 2014; Sun et al., 2015; Yan et al., 2017), and no studies have evaluated the challenges for large-scale production. To date, the methods presented in the literature are diverse and rely on the use of different expression vectors, strains and a wide variety of culture conditions (Anders and Jinek, 2014; Gagnon et al., 2014; Zuris et al., 2015; Wang et al., 2018; Rajagopalan et al., 2018). Cas9 is a large protein derived from the bacterium *Streptococcus pyogenes* that contains 1368 amino acids and has a molecular weight of 158 kDa (Gagnon et al., 2014). Although the protein is suitable for heterologous expression using *E. coli* (Jinek et al., 2014; Ramakrishna et al., 2014), its large size and the presence of 137 rare codons in the corresponding gene may result in significant challenges during recombinant production. Many of the protocols used for Cas9 expression make use of long culture and induction times at low temperature (18 °C) using complex media (Gagnon et al., 2014; Liu et al., 2015; Yan et al., 2017), conditions that usually indicate difficulties during protein expression, and that are not ideal for large scale production.

Owing to the importance and potential of the Cas9 protein and the scarcity of studies published in the literature that have evaluated its production, we believed that it was important to carry out a systematic study to improve the understanding of the expression of Cas9 in *E. coli* and to develop a feasible and scalable production process. Here, we evaluate the influence of the bacterial strain and the culture conditions (use of complex and defined medium, temperature of induction, post induction time, and the use of shaker flasks and bioreactors) on the production of Cas9 protein in *E. coli*.

2. Materials and methods

2.1. Plasmids, bacterial strains and culture media

The *E. coli* strains BL21 (DE3) and BL21 (DE3) Rosetta were transformed with the expression vector pET-28b-Cas9-His and stored at −80 °C. The pET-28b-Cas9-His vector was obtained from Addgene Plasmid Repository (#47327; http://n2t.net/addgene:47327; RRID:Addgene_47327, deposited by Professor Alex F. Schier). The vector contains the Cas9 gene (4104 bp) with a histidine tag in its c-terminus, a kanamycin resistance gene and a T7 promoter (Gagnon et al., 2014). The BL21(DE3) containing the plasmid pET-28b-Cas9-His was also cotransformed with an empty plasmid pACYCDuet-1 (Merck Millipore/Novagen, USA) and used as a control to verify the effect of the chloramphenicol resistance gene expression on the Cas9 protein expression. Similarly to pRARE plasmid (4694 bp) present in Rosetta strain, the pACYCDuet-1 (4008 bp) plasmid carries the P15A origin of replication and the chloramphenicol resistance gene. The presence of both plasmids in cotransformed clones was verified by pDNA extraction followed by agarose gel electrophoresis. Finally, the pVAX1GFP plasmid (3697 bp) was used for the Cas9 protein activity assay. The plasmid was designed for DNA vaccine development and contains the *GFP* gene (Azzoni et al., 2007). All bacterial strains were freshly transformed, cultured in LB medium until mid-exponential phase (OD_{600nm} of ~0.8), and the cells were stored in aliquots (15% glycerol) at −80 °C, composing the Master Cell Banks.

Two different kinds of media were used in this work: a complex Lysogeny broth (LB medium) and a defined medium (high density fermentation - HDF - medium). The LB medium contained tryptone (10.0 g/L; Liofilchem Inc., Italy, K25-611004), yeast extract (5.0 g/L; LabSynth Ltda., Brazil, E0002.08.AG) and NaCl (10.0 g/L; LabSynth Ltda., Brazil, ACS grade). The defined medium contained (per liter) glucose (10.0 g), KH_2PO_4 (13.3 g), $(NH_4)_2HPO_4$ (4.0 g), $MgSO_4.7H_2O$ (1.2 g), citric acid (1.7 g), EDTA (14.1 mg), Fe(III) citrate (100.8 mg), thiamine HCl (4.5 mg), $CoCl_2.6H_2O$ (2.5 mg), $MnCl_2.4H_2O$ (15.0 mg), $CuCl_2.2H_2O$ (1.5 mg), H_3BO_3 (3.0 mg), $Na_2MoO_4.2H_2O$ (2.1 mg), and $Zn(CH_3COO)_2.2H_2O$ (33.8 mg). The pH was adjusted to 7.0 by the addition of 25% (v/v) NH_4OH solution. A final concentration of 30 µg/mL kanamycin sulfate was used in the medium (LB or HDF) when growing the strains harboring the pET-28b-Cas9-His vector. Chloramphenicol (34 µg/mL) was also included for cultivations using the BL21(DE3) Rosetta strain and the control BL21(DE3) strain harboring the pET28b-Cas9-His and pACYCDuet-1 vectors. The chemicals used in this work for medium and buffer preparation were purchased from Sigma-Aldrich (USA, at least ACS grade). The exceptions were Tris-HCl (GE Healthcare, Sweden, code 17-1321-01) and the chemicals H_3BO_3, $Na_2MoO_4.2H_2O$, and NH_4OH (LabSynth Ltda., Brazil, ACS grade).

2.2. Cell growth and the expression of recombinant Cas9 protein in E. coli

The inoculums were prepared by transferring 50 µL of cell suspension that had been stored at −80 °C (Master Cell Bank aliquots, item 2.1) to a 250 mL Erlenmeyer flask containing 50 mL of LB or HDF medium. The cells were incubated at 37 °C and 250 rpm for 16 h. A portion of this culture broth was then inoculated into a shake flask or a bioreactor at an initial OD_{600nm} of 0.2.

For the shake flask experiments, the cultures were incubated in 500 mL Erlenmeyer flasks containing 100 mL of culture medium (LB or HDF medium) at 37 °C and 250 rpm until the OD_{600nm} reached 0.6-0.8 for the LB medium and 1.2–1.4 for the HDF medium. The culture was induced by the addition of IPTG (final concentration of 0.5 mM), and the temperature was decreased to 25 °C for 4 h. The optimization of the induction conditions was carried out in HDF medium. When the OD_{600nm} reached 1.2–1.4, the culture was induced with IPTG (0.5 mM) at different temperatures (18, 25, 30 and 37 °C) for 4 h. At the optimal temperature of induction (30 °C), different induction times (0, 2, 4, 6, 8, 18 and 24 h) were evaluated.

The batch bioreactor cultivation was performed in a BioFlo III Benchtop Bioreactor (Eppendorf/New Brunswick Scientific, USA) containing 2.0 L of HDF medium. The culture was maintained at 37 °C. The dissolved oxygen (DO) level was maintained at 30% of saturation and controlled by supplying air or pure oxygen with an agitation rate that ranged from 200 to 800 rpm. The pH value was maintained at 7.0 by the automatic addition of 25% (v/v) NH_4OH solution. The culture was induced by the addition of IPTG (0.5 mM), and the temperature was decreased to 30 °C for 8 h. Samples (90 mg of cells for the purification process and 0.7 mg of cells for the SDS-PAGE analysis) from all experiments were collected, and the cells were harvested by centrifugation (8000*g*, 10 min, 4 °C). The pellet was stored at −20 °C.

2.3. Purification of recombinant Cas9 protein

After harvesting, the pellet (90 mg of cells) was resuspended in 20 mL of adsorption buffer (20 mM Tris-HCl pH 8.0, 30 mM imidazole, and 500 mM NaCl). The cells were lysed in an icewater bath with 8 cycles of 30 s of sonication followed by a 30 s pause. The crude extract (supernatant) was obtained by centrifugation (12000*g*, 20 min, 17 °C), and the Cas9 protein was purified by chromatography or analyzed by SDS-PAGE and densitometry.

The Cas9 protein purification was performed by gravity in a column containing 1 mL of Ni-NTA-agarose resin (Qiagen, USA) equilibrated with adsorption buffer. Ten milliliters of cell lysate was added to the column, followed by washing with 20 mL of adsorption buffer. The purified Cas9 protein was eluted with 5 mL of adsorption buffer containing 500 mM imidazole and dialyzed in dialysis buffer (20 mM Hepes

pH 7.5, 150 mM KCl, 1 mM DTT, and 10% glycerol) overnight. The purified Cas9 protein was analyzed by SDS-PAGE and quantified by Bradford assays and densitometry.

2.4. Assay of Cas9 protein activity

The templates for RNA *in vitro* transcription that encoded a T7 promoter followed by the gRNA sequence were synthesized by IDT (Integrated DNA Technologies, USA) along with sgRNA containing a 20-base GFP targeting sequence and a cgRNA (control guiding RNA) that does not target GFP or any gene in the pVAX1GFP plasmid (Table 1) (Cho, 2013). The sgRNA and cgRNA were transcribed *in vitro* with a GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The pVAX1GFP plasmid containing the GFP gene was linearized with the XbaI enzyme (Thermo Fisher Scientific, USA) and purified by the Illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare, Sweden). The Cas9 protein activity assay was carried out in 20 µL of NEB Buffer 3. The purified Cas9 protein (50 nM) was incubated with gRNA (50 nM) for 10 min at 25 °C, followed by the addition of linearized pVAX1GFP plasmid (5 nM). After digestion for 1 h at 37 °C, the solution was incubated for 10 min at 65 °C. The DNA fragmentation was analyzed by 1% agarose gel electrophoresis.

Table 1
Sequences of the DNA oligonucleotides used for RNA *in vitro* transcription. The T7 promoter sequence is presented in bold. The target DNA sequence is underlined. The non-target sequence is presented in italic.

sgRNA	5'-**GTTTTTTTTAATACGACTCACTATA**GGGCGAGGAGCTGTTCA CCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC GTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT-3'
cgRNA	5'-**TTTTTTTTAATACGACTCACTATA**GGGTAACCGTGCGGTCG TAC*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC* *CGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT-3'*

2.5. Analytical methods

Cell growth was monitored by measuring the OD_{600nm} with a spectrophotometer (Quimis, Brazil). The cell concentration was estimated with a calibration curve for the dry cell weight that had been previously obtained. The glucose concentration was measured using a YSI 2700 SELECT analyzer (Yellow Springs Instruments, Marshall Scientific, USA). The acetate concentration was measured by HPLC (Ultimate 3000, Thermo Fisher Scientific Inc., USA) using an Aminex HXP-87H column (Bio-Rad, USA). The mobile phase contained 5.0 mM sulfuric acid, and the flow rate was 0.5 mL/min at 50 °C. The total protein concentration was quantified using the Bradford method, and bovine serum albumin was used as a reference protein (Bradford, 1976). The recombinant protein that was expressed and had accumulated in cell lysates was analyzed by SDS-PAGE and Western blotting. The sample pellets (0.7 mg of cells) were suspended in sample buffer and heated to 100 °C for 10 min (Laemmli, 1970). The samples were loaded in 12% polyacrylamide gels and stained with Coomassie Brilliant Blue. The Western blot analysis was carried out as described by Towbin et al. (1979). The proteins were separated in a 10% polyacrylamide gel and transferred to a nitrocellulose membrane(0.2 µm; BioRad, USA). A mouse monoclonal anti-His-Tag antibody (#MA1-21315, Thermo Fisher Scientific Inc., USA) and an anti-mouse IgG-peroxidase antibody produced in goat (A4416, Sigma-Aldrich, USA) were used as the primary and secondary antibodies. The protein bands were detected by using the chemiluminescent Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare, USA). The Cas9 protein was quantified by densitometry using ImageJ software (U. S. National Institutes of Health, USA) and normalized to the 50 kDa protein band within the standard marker (Larentis et al., 2011). The relative purity of the Cas9 protein after the elution step was determined as a percentage of the intensity of the Cas9 protein band compared to the sum of the intensities of all other bands in the same lane (Carvalho et al., 2012).

2.6. Cas9 mRNA analysis by qPCR

Cas9 mRNA synthesis was analyzed by quantitative real-time PCR (qPCR). The sequences of the primers used are presented in Table 2. The *E. coli* BL21(DE3) and BL21(DE3) Rosetta strains (with and without pET28b-Cas9His) were cultured in 100 mL of LB media at 37 °C and 250 rpm. When the OD_{600nm} reached 0.6-0.8, the cultures were induced with 0.5 mM IPTG at 30 °C for 4 h. Samples (1 mg of cells) were collected, and the cells were harvested by centrifugation (8000*g*, 10 min, 4 °C). The RNA extraction was carried out with the PureLink™RNA Mini Kit (Invitrogen, USA) according to the manufacturer's instructions. The total RNA concentration was determined using a NanoDrop (Thermo Fisher Scientific Inc., USA) at 260 nm. Samples with the same amount of total RNA were treated with DNase I (Promega™, USA) at 37 °C for 50 min, and the DNase was inactivated by adding EGTA and incubating the sample at 65 °C for 10 min. The cDNA synthesis was carried out with the M-MLV Reverse Transcriptase kit (Invitrogen, USA) according to the manufacturers' instructions. Then, the cDNA samples were quantified by qPCR using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific Inc., USA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems™-Thermo Fisher Scientific Inc., USA). The cycle conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 54 °C for 20 sand 60 °C for 20 s. The formation of nonspecific products was evaluated by measuring the melting curves. The number of copies in each sample was determined on the basis of a standard curve obtained from pET28bCas9His. The relative values were normalized to the highest number of copies measured in the sample from the BL21(DE3) strain containing pET28bCas9His after induction.

Table 2
Sequences of the primers used for Cas9 mRNA qPCR analysis.

Primer	Sequence (5'→3')	Tm (°C)*
(1) Reverse_cDNA	CTG CTC TCC AGA CAG GAA TGC T	59,5
(2) Forward_qPCR	GCG AGT GAA CAC GGA GAT CAC C	61,4
(3) Reverse_qPCR	ACT TCT CAG GCA GTT GCT GTC T	57,7

* Melting temperature.

3. Results and discussion

3.1. Cas9 protein expression using shake flasks

Escherichia coli has been widely used as a platform for the expression of recombinant proteins due to the extensive body of knowledge that already exists about its genetics and metabolism, the availability of efficient molecular tools and the easy manipulation and rapid cell growth of *E. coli*. In many cases, the expression of the protein of interest occurs at a high level within a few hours after induction (Demain and Vaishnav, 2009; Tripathi, 2016). Moreover, depending on the properties of the target protein, a variety of *E. coli* strains are available that can be used to overcome challenges that may arise during recombinant protein expression (Tripathi, 2016).

The Cas9 protein (the protein of interest in this work) is a large protein with 1390 amino acids and a molecular weight of 158 kDa (Jinek et al., 2014). It is a monomeric endonuclease from the bacterium *Streptococcus pyogenes*, which allows *E. coli* to serve as an expression system (Hwang et al., 2013). However, the coding sequence of the Cas9 protein contains a significant number of codons that are rare in *E. coli* (Table 3).The presence of rare codons can be a drawback for recombinant protein expression in *E. coli* due to the low availability of some rare tRNAs during translation, which may significantly decrease the protein yield or even lead to the expression of truncated proteins (Berlec and Strukelj, 2013; Feng et al., 2015; Aguirre-López et al., 2017). One of the most commonly used *E. coli* strains for the expression of genes containing rare codons is BL21(DE3) Rosetta. This *E. coli* strain harbors a plasmid (pRARE) that encodes rare tRNAs. Although it has not been thoroughly

established that increased protein production is due to tRNA compensation, the literature indicates that Rosetta strain tends to increase the success in the expression and purification of human recombinant proteins containing rare codons (Tegel et al., 2010).

Table 3
Number and type of *Cas9* rare codons in *E. coli* according to the results of the Caltor program (http://people.mbi.ucla.edu/sumchan/caltor.html).

Amino acid	Rare codon	Number of codons in *Cas9* sequence
Arginine	CGA	9
	CGG	20
	AGG	21
	AGA	15
Glycine	GGA	23
	GGG	15
Isoleucine	AUA	8
Proline	CCC	18
Threonine	ACG	8

As indicated by the literature, optimized *E. coli* strains, including BL21(DE3) Rosetta, have been preferred for Cas9 protein expression (Ramakrishna et al., 2014; Gagnon et al., 2014; Zuris et al., 2015; Sun et al., 2015; DeWitt et al., 2017). However, the expression conditions used differed greatly, and usually, there were no concerns about productivity and scale-up viability. Furthermore, these studies used complex culture mediums, low temperatures of induction and long induction times, which usually indicate the presence of difficulties and correlate with low levels of Cas9 protein expression. In addition, none of these studies was performed with more stringent control of bacterial growth in bioreactors.

Here, Cas9 protein expression using BL21(DE3) and BL21(DE3) Rosetta as host strains was first evaluated in shake flasks with 250 mL of Lysogeny broth (LB). The induction of protein expression was performed using 0.5 mM IPTG (1.6 mmol IPTG/g CDW) at 30 °C for 4 h. Under these conditions, recombinant protein expression in BL21(DE3) Rosetta was much lower than that in BL21(DE3). In fact, the expression of Cas9 in the Rosetta strain could only be detected by Western blotting analysis at the elution peak after capture in a Ni-NTA-packed bed column (Fig. 1). Several other culture conditions for the Rosetta strain were tested, using low temperatures of cell growth and induction, including the use of auto-induction medium, and these always resulted in barely detectable Cas9 expression levels (data not shown). On the other hand, the expression of Cas9 protein in the regular BL21(DE3) strain was easily detected in crude cell extracts, and significant amounts of protein could be purified in a single Ni-NTA chromatography procedure (Fig. 1).

Differently from the regular BL21(DE3) strain, Rosetta cells are grown in the presence of chloramphenicol to maintain the pRARE plasmid. In order to verify possible metabolic effects caused by the presence of chloramphenicol resulting in the decrease of Cas9 protein expression, we compared Rosetta strain with a control BL21(DE3)

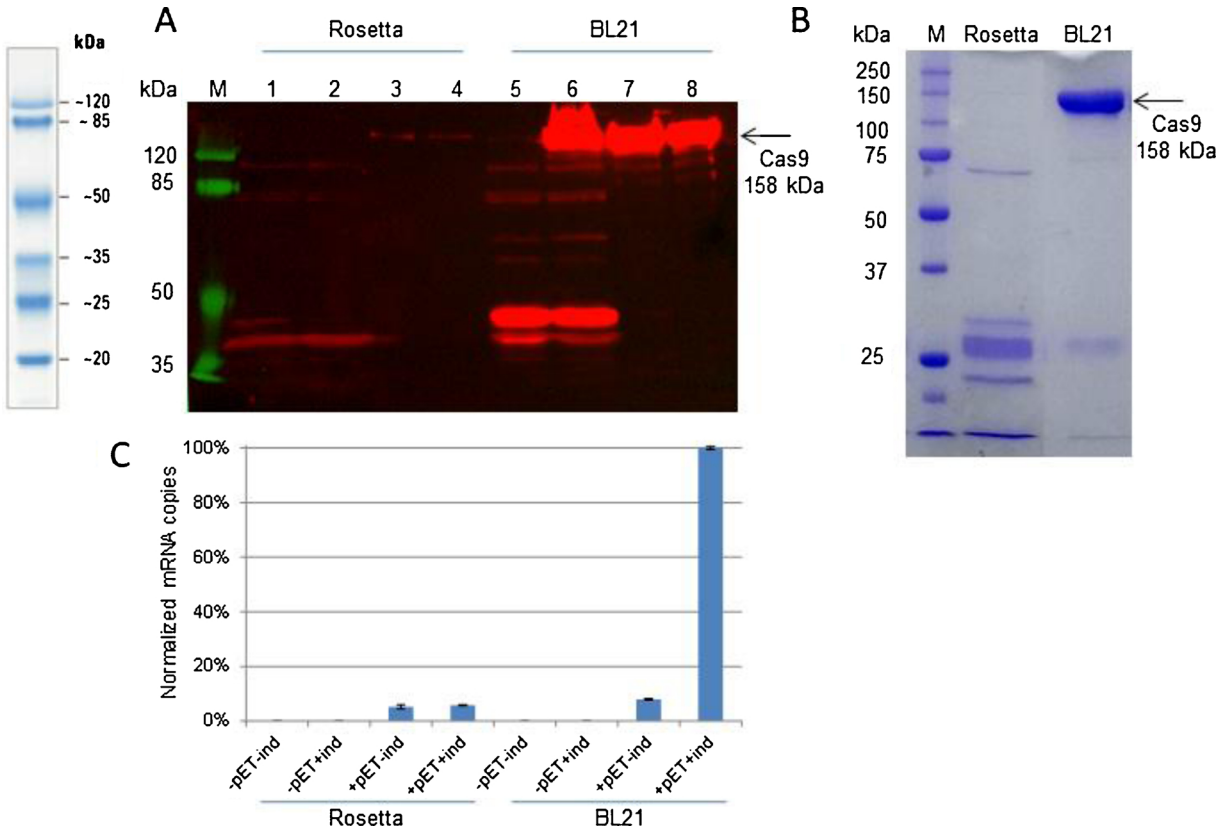

Fig. 1. Evaluation of Cas9 protein expression in BL21(DE3) and BL21(DE3) Rosetta cells induced with 0.5 mM IPTG at 30 °C for 4 h in LB medium. (**A**) Western blot analysis of Cas9 expression. Legend: M: prestained protein MW marker; 1: BL21(DE3) Rosetta cell lysate before induction; 2: BL21(DE3) Rosetta cell lysate at 4 h post induction; 3: fraction 2 from the elution step for Cas9 protein expressed in BL21(DE3) Rosetta; 4: fraction 3 from the elution step for Cas9 protein expressed in BL21(DE3) Rosetta; 5: BL21(DE3) cell lysate before induction; 6: BL21(DE3) cell lysate at 4 h post induction; 7: fraction 2 from the elution step for Cas9 protein expressed in BL21(DE3); 8: fraction 3 from the elution step for Cas9 protein expressed in BL21(DE3). (**B**) SDS-PAGE analysis of Cas9 protein purified using Ni-NTA affinity chromatography. M: unstained protein standards; Rosetta and BL21: elution peaks from the Ni-NTA column. (**C**) Relative numbers of Cas9 mRNA copies in samples from BL21(DE3) and BL21(DE3) Rosetta with or without the pET28bCas9His plasmid (+pET; -pET) and induced or not induced (-ind; +ind) that are normalized to the highest copy number in the BL21(DE3) strain sample containing the pET28bCas9His plasmid after induction. The error bars indicate the standard deviation of triplicate measurements.

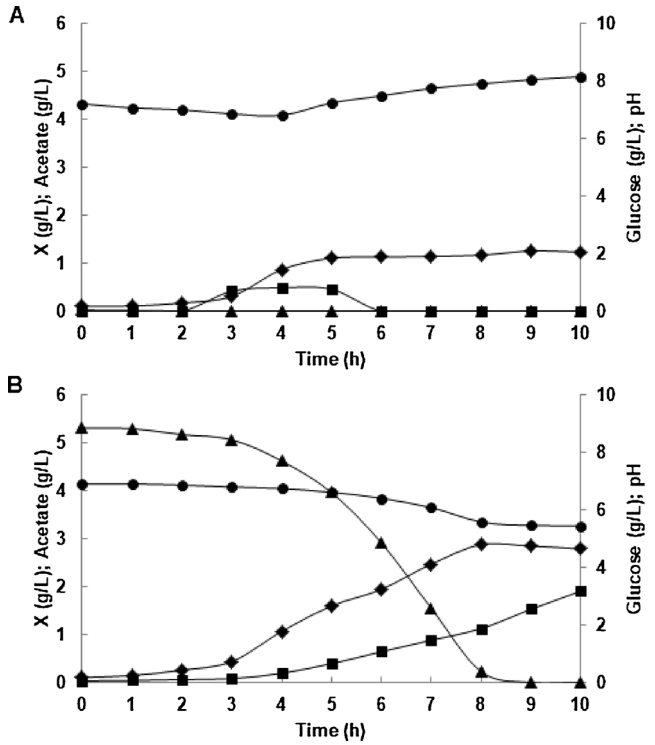

Fig. 2. Growth kinetics of *E. coli* BL21(DE3) in shake flasks at 37 °C in (**A**) LB medium and; (**B**) HDF medium. (—◆—) X, cell concentration (g/L); (—■—) acetate (g/L); (—▲—) glucose (g/L); (—●—) pH. No induction of protein expression was performed in these experiments.

harboring the plasmid pACYCDuet-1. Similarly to pRARE plasmid (4694 bp), the pACYCDuet-1 (4008 bp) plasmid carries the P15A origin of replication and the chloramphenicol resistance gene. An experiment was performed simultaneously comparing the Cas9 expression level presented by the *E. coli* BL21(DE3), BL21(DE3) Rosetta and the control BL21(DE3) pACYCDuet-1 (Figs. 1S and 2S, Supplementary data). Comparing to BL21(DE3), the Rosetta strain presented very low levels of Cas9 expression, while the control BL21(DE3) pACYCDuet-1 presented a reduction of 35% on Cas9 expression (final Cas9 concentrations of 18.4 and 11.9 mg·L^{-1} for BL21(DE3) and BL21(DE3) pACYCDuet-1, respectively) with similar levels of biomass concentration at the end of growth. These control experiments indicated that the presence of chloramphenicol reduced the Cas9 protein expression, although not at the very low levels verified using the Rosetta strain.

The low expression level presented by the Rosetta strain was an unexpected result because the Cas9 protein has a large number of rare codons in *E. coli*. In the literature, there is a hypothesis that proposes a possible role of rare codons in the kinetics of protein translation and folding (Berlec and Strukelj, 2013; Feng et al., 2015; Aguirre-López et al., 2017). This could be an explanation for the low expression of Cas9 in the Rosetta strain, especially considering the large size and complexity of this nuclease. Tegel et al. (2010) also observed some cases of lower levels of protein expression in the BL21(DE3) Rosetta strain than in the BL21(DE3) strain, although the Rosetta produced better results in general. One possibility raised by these authors was that the pRARE plasmid resulted in an extra metabolic burden. Interestingly, most of the reported Cas9 expression conditions in the Rosetta strain found in the literature involved low temperatures of induction, usually at 18 °C (Gagnon et al., 2014; Sun et al., 2015; DeWitt et al., 2017), which is indicative of a decrease in cell metabolism.

In an attempt to understand the reasons for the low level of Cas9 protein expression when using the Rosetta strain, qRT-PCR was carried out to compare the Cas9 mRNA levels in the BL21(DE3) and BL21(DE3) Rosetta strains before and after induction with IPTG. Surprisingly, the results indicated that the reduced Cas9 protein expression in the Rosetta strain was related to the low level of transcribed Cas9 mRNA (Fig. 1c). We postulate that the large size of the Cas9 mRNA and the metabolic stress due to the presence of the extra pRARE plasmid in the Rosetta strain could affect the transcription rate of the Cas9 gene. In line with this conclusion, Søgaard and Nørholm (2016) observed that several DasherGFP protein variants they tested exhibited a significant drop in production in the presence of the pLysSRARE2 plasmid in different strains and under different growth conditions. This study indicates that the presence of additional plasmids containing extra copies of tRNA genes may lead to negative metabolic side effects that reduce recombinant protein expression levels.

Our next step was to evaluate the expression of the Cas9 protein in the regular BL21(DE3) strain to determine the factors affecting expression that may result in higher protein production. The cells were cultivated in shake flasks in complex Lysogeny broth (LB) and HDF medium to evaluate the cell growth kinetics and Cas9 protein expression. The first step was evaluating the growth kinetics without induction of protein expression. Fig. 2 shows the cell growth (biomass), glucose consumption and acetate formation in cultures grown in LB and HDF media at 37 °C and 250 rpm. The maximum specific growth rate in LB medium (0.68 h^{-1}) was similar to that in HDF medium (0.61 h^{-1}).

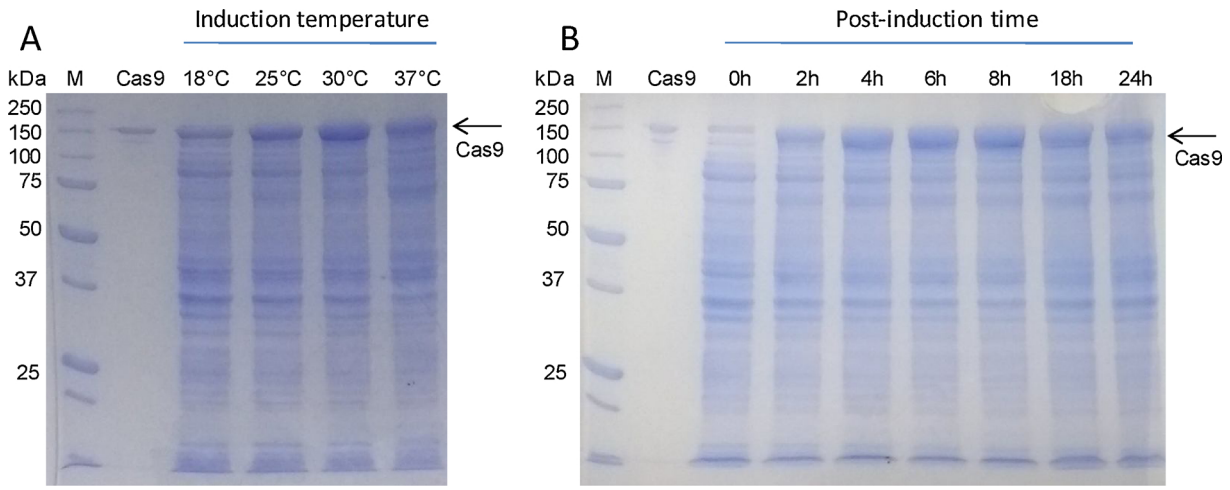

Fig. 3. Evaluation of the effects of induction temperature and post induction time on the expression of Cas9 protein in *E. coli* in HDF medium (0.36 mmol IPTG/g CDW). (**A**) SDS-PAGE analysis of the expression of Cas9 protein at different induction temperatures (18, 25, 30 and 37 °C); (**B**) Expression of Cas9 protein at 30 °C and at different post induction times (0, 2, 4, 6, 8, 18 and 24 h); M: unstained protein standards; Cas9: Ni-NTA-purified Cas9 protein.

Table 4
Recombinant Cas9 protein expression data using BL21(DE3) strain grown at the different conditions tested in this work.

Experimental setup	Cas9 concentration ($mg{\cdot}L^{-1}$)	Cell concentration (g $CDW{\cdot}L^{-1}$)	Cultivation time (h)	Cas9 volumetric productivity ($mg{\cdot}L^{-1}{\cdot}h^{-1}$)	Cas9 specific production rate ($mg.g\ CDW^{-1}{\cdot}h^{-1}$)
Shake flask					
LB medium	13.6	1.02	7.5	1.81	1.8
HDF medium	35.0	2.64	7.5	4.67	1.8
Induction temperature (°C)					
18	12.1	1.76	7.5	1.61	0.91
25	29.6	2.63	7.5	3.94	1.50
30	45.1	3.02	7.5	6.01	1.99
37	30.8	3.58	7.5	4.10	1.15
Post-induction time (h) at 30 °C					
0	2.40	1.30	3.5	0.68	0.53
2	22.0	2.27	5.5	4.00	1.76
4	52.7	3.13	7.5	7.02	2.24
6	74.3	4.01	9.5	7.82	1.95
8	96.4	4.80	11.5	8.38	1.74
18	98.4	6.65	21.5	4.57	0.68
24	75.6	6.70	27.5	2.75	0.41
Bioreactor					
Induction 1*					
0h post-induction	2.0	0.51	3	0.67	1.31
8 h	131	2.27	11	11.9	5.23
Induction 2**					
0h post-induction	8.4	1.84	5	1.68	0.91
4 h	214	4.23	9	23.8	5.61
6 h	420	5.34	11	38.2	7.15
8 h	320	5.33	13	24.6	4.62

* Reported in the article as induction at the beginning of the exponential phase.
** Induction at the middle of the exponential phase.

However, the cell density in HDF medium was significantly higher than that in LB medium (2.8 and 1.2 g $CDW{\cdot}L^{-1}$, respectively). The overall substrate-to-cell yield in HDF medium was 0.30 g CDW/g glucose.

LB complex medium has been widely reported in the literature for *E. coli* growth and the expression of recombinant proteins at small scales (Losen et al., 2004). This medium contains a low concentration of carbon sources (carbohydrates), which forces cells to use amino acids as a carbon source, and this usually leads to the production of ammonium, which increases the culture pH (Sezonov et al., 2007). However, the use of the HDF defined medium is indicated when the intention is to obtain high cell densities and the enhanced production of recombinant proteins (Silva et al., 2013; Carvalho et al., 2012). Nevertheless, the use of defined media for the production of therapeutic proteins is preferred due to the resulting improvement in process control and reproducibility and the simplification of downstream processing (Huang et al., 2012). The composition of the HDF medium was adapted from Seeger et al. (1995) and includes glucose as a carbon source, although different carbon sources may also be used (e.g., glycerol).

One possible outcome of the presence of excess carbon sources in the medium is overflow metabolism (the Crabtree effect) and, consequently, the formation of acetate (Akesson et al., 2001). Acetate is a by-product of *E. coli* metabolism that reduces the growth rate of the culture and decreases recombinant protein production (Kleman and Strohl, 1994). The formation of acetate also occurs as a result of limited oxygen levels, reducing cell growth (Akesson et al., 2001). The measurement of BL21(DE3) cell growth in HDF medium in shake flasks, as shown in Fig. 2b, indicates almost linear cell growth over time, which is likely a result of limited oxygen levels, the fast consumption of glucose, and the significant formation of acetate.

The expression of recombinant Cas9 after induction was also evaluated using 0.5 mM IPTG and induction temperature of 25 °C for 4 h. Inductions were performed approximately in the middle of the growth phase with inducer loads of 0.72 mmol IPTG/g CDW and 1.6 mmol IPTG/g CDW for HDF and LB medium, respectively. The results indicated a similar specific recombinant protein production rate when using HDF and LB medium (1.8 $mg{\cdot}g\ CDW^{-1}{\cdot}h^{-1}$), although a higher Cas9 volumetric productivity ($mg{\cdot}L^{-1}{\cdot}h^{-1}$) could be achieved in HDF due to the higher cell density at the end of culturing (Table IV).

The next step was to evaluate the effects of induction temperature and post induction time on the expression of Cas9 protein in the BL21(DE3) strain in HDF medium. These assays were carried out in shake flasks, and induction occurred during the exponential growth phase via the addition of 0.5 mM IPTG when an OD_{600nm} of approximately 1.4 was reached (0.36 mmol IPTG/g CDW). The cells were collected after 4 h of induction, as described previously. Four different induction temperatures (18, 25, 30 and 37 °C) were evaluated. The cell lysate corresponding to each condition was analyzed by SDS-PAGE and quantified by densitometry. The results are presented in Fig. 3 and Table 4.

The results obtained for the expression of Cas9 protein at 37 °C and 30 °C indicated that, although the final cell density was higher at 37 °C, the *E. coli* presented a lower Cas9 specific production rate at 37 °C than at a lower induction temperature (30 °C), as seen on Table 4. Therefore, the results indicate that the best induction temperature for the production of Cas9 protein was 30 °C, which yielded a final volumetric productivity of 6.01 $mg{\cdot}L^{-1}{\cdot}h^{-1}$.

Because a higher Cas9 specific production rate was obtained at 30 °C, the post induction time was also evaluated at this temperature. It was observed that a maximum of protein specific production rate was obtained at 4 h post induction (2.24 $mg{\cdot}g\ CDW^{-1}{\cdot}h^{-1}$) although volumetric productivity continued to increase up to 8 h post induction (8.38 $mg{\cdot}L^{-1}{\cdot}h^{-1}$), as a result of the increase in total cell concentration.

3.2. Expression of Cas9 protein in a bioreactor

To evaluate the production of recombinant Cas9 protein on a larger scale, batch fermentations were carried out using a 2 L bioreactor containing HDF medium and 10 g/L glucose as the carbon source. The expression of Cas9 protein occurred under conditions previously used in shake flasks, in which growth occurred at 37 °C followed by induction at 30 °C for 8 h. Some of the advantages of using a bioreactor are the ability to maintain a constant pH and the avoidance of oxygen-limited

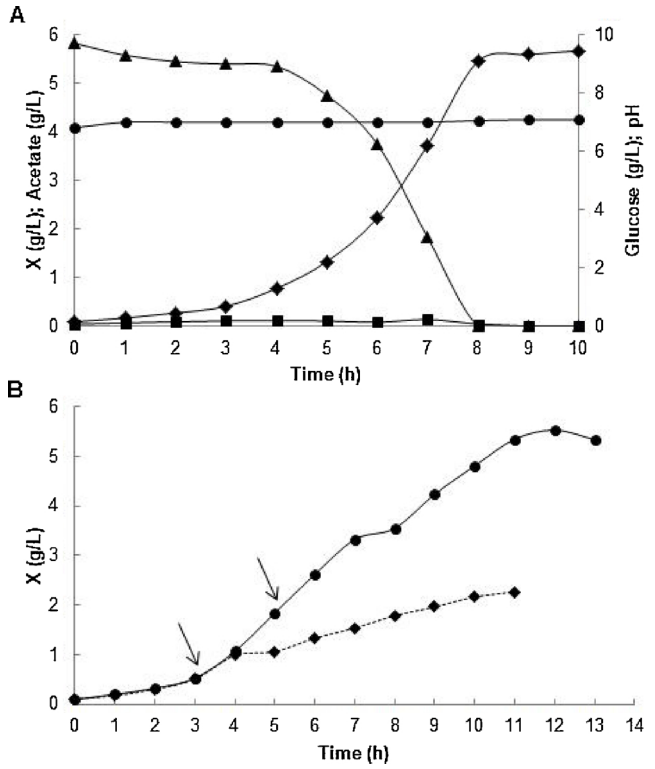

Fig. 4. Growth of *E. coli* BL21(DE3) in a bioreactor. (**A**) Growth kinetics of BL21(DE3) in HDF medium at 37 °C (no induction); (—◆—) X: cell concentration (g/L); (—■—) acetate (g/L); (—▲—) glucose (g/L); (—●—) pH. (**B**) Comparison of cell growth during Cas9 protein expression when two different times of induction were used: (---◆---) beginning of the exponential phase; (—●—) middle of the exponential phase. Induction was performed at 30 °C with 1.0 mmol IPTG/g CDW and 0.275 mmol IPTG/g CDW, for induction at the beginning and middle of the exponential phase, respectively. The arrows indicate the times of induction.

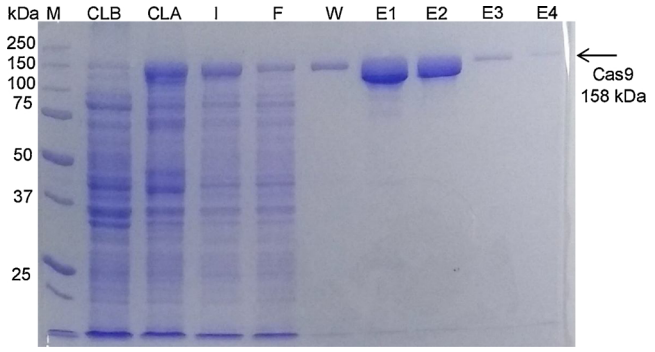

Fig. 5. SDS-PAGE analysis of Cas9 protein samples obtained during purification on a Ni-NTA-agarose column. M: Precision Plus Unstained Protein Standards (Bio-Rad, USA); CLB: cell lysate before induction; CLA: cell lysate after induction; I: feed solution; F: flow-through; W: wash solution; E1 to E4: fractions from the elution step.

Table 5
Recovery and relative purity of Cas9 protein obtained during each Ni-NTA purification step. The relative purity was estimated by gel densitometry.

Purification step	Total protein (mg)	Recovery (%)	Relative purity (%)
Feed	2.8	—	—
Washing	2.3	82	—
Elution	0.8	28	96

conditions (Huang et al., 2012). The control of these parameters is important during cell growth and recombinant protein expression in *E. coli,* since extreme pH can cause metabolic stress and a lack of dissolved oxygen can be a significant limiting factor for cell growth (Hanning and Makrides, 1998; Phue and Shiloach, 2005).

Initially, an experiment was carried out to understand the growth kinetics of *E. coli* BL21(DE3) in HDF medium at 37 °C in a bioreactor (Fig. 4A). The maximum cell concentration obtained was 5.6 g CDW·L^{-1}, and the maximum specific growth rate (μ_{max}) was 0.53 h^{-1}. The substrate-to-cell yield factor ($Y_{x/s}$) was 0.55 g of cells/g of glucose. Although the maximum specific growth rate in the bioreactor was lower than that in the shake flasks (0.61 h^{-1}), the exponential growth phase in the bioreactor was considerably longer (7 h), resulting in higher values for the final cell concentration and $Y_{x/s}$. In the bioreactor, the glucose consumption was clearly a limiting factor for cell growth (Fig. 4A), and the control of dissolved oxygen favored cell growth and substrate conversion in cells, since it was not a limiting factor for the process.

Cas9 protein expression was also evaluated in two different experiments to verify whether induction at different stages of cell growth can affect production. The inductions were carried out in the beginning and in the middle of the exponential phase (indicated by arrows in Fig. 4B), with inducer loads of 1.0 mmol IPTG/g CDW and 0.275 mmol IPTG/g CDW, respectively. The results shown in Fig. 4B indicate a strong inhibition of cell growth after induction at the beginning of the exponential phase. It was described that an over-supply of inducer negatively affects the host cell metabolism due to the strength of the T7 expression system (Striedner et al., 2003). The Cas 9 specific production rate and volumetric productivity after 8 h of induction were 5.23 mg·g $CDW^{-1}·h^{-1}$ and 11.9 $mg·L^{-1}·h^{-1}$, respectively (Table 4). When induction was carried out in the middle of the exponential phase, it was verified that the Cas9 specific production rate and volumetric productivity reached a maximum after 6 h of induction (7.15 mg·g $CDW^{-1}·h^{-1}$ and 38.2 $mg·L^{-1}·h^{-1}$, respectively, Table 4). These results confirmed that inducer should be supplied in a certain ratio to biomass along with the increase of biomass to avoid metabolic overload, as proposed by Striedner et al. (2003); however, in our work, optimization of inducer supply was not performed.

3.3. Cas9 protein purification by IMAC and the Cas9 activity assay

Cas9 protein was also purified from cell crude extracts using immobilized metal-ion affinity chromatography (IMAC, in Ni-NTA resin) (Fig. 5) to take advantage of the presence of a His-tag at the C-terminus of the recombinant Cas9 protein. The purification was evaluated by SDS-PAGE and densitometry (using ImageJ software). The gel shows the presence of a 158 kDa band in the fractions collected during the elution step (after the addition of imidazole), which corresponds to the Cas9 protein. The overall recovery of the Cas9 protein during the purification procedure was 28%, and the relative purity of Cas9 in the first fraction from the elution step was estimated to be 96% (Table 5). However, the low recovery can be attributed to resin saturation during loading, since the purification process was not optimized in this study.

The activity of the purified Cas9 protein produced in both LB and HDF medium was tested by *in vitro* DNA cleavage. The recombinant Cas9 protein and a sgRNA containing the green fluorescent protein (GFP) target sequence were incubated with linearized plasmid DNA encoding the *GFP* gene. The results in Fig. 6 show that the complexes formed by the Cas9 protein and sgRNA were able to cleave the plasmid at the *GFP* gene sequence.

4. Conclusions

Here, we evaluated recombinant Cas9 nuclease expression in the *E. coli* BL21(DE3) and BL21(DE3) Rosetta strains using complex and defined media. Surprisingly, the Rosetta strain exhibited strikingly low

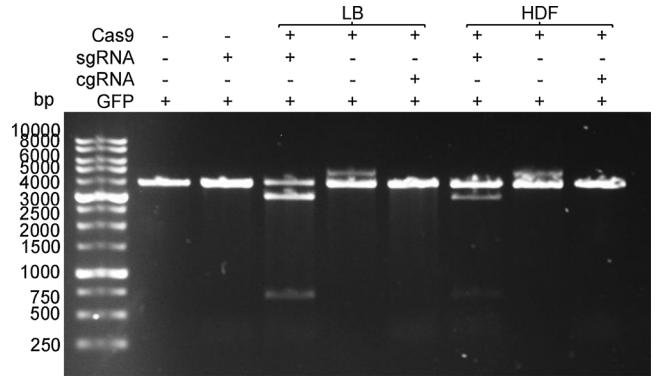

Fig. 6. Activity assay of Cas9 protein produced in LB and HDF media using linearized pVAX1GFP plasmid DNA (3697 bp) as a substrate. Only a complex of Cas9 and sgRNA could digest the plasmid DNA.

recombinant Cas9 protein expression in all media and conditions tested. The determination of the Cas9 mRNA levels in both strains, both before and after induction, using IPTG indicated that there was a significant transcription bottleneck in the Rosetta strain, although a detailed study of the causes of this limitation was not performed in this study. Overall, the BL21(DE3) strain cultures could be optimized and scaled-up, indicating that active Cas9 nuclease could be easily overexpressed in a simple synthetic medium (HDF) using a bioreactor operated in batch mode.

Declaration of Competing Interest

The authors declare no financial or commercial conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2019.09.012.

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