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CRISPR/Cas9 technology enables the development of the filamentous ascomycete fungus *Penicillium subrubescens* as a new industrial enzyme producer

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ABSTRACT

Penicillium subrubescens is an ascomycete fungus with an enriched content of specific carbohydrate-active enzyme families involved in plant biomass degradation, which makes this strain a promising industrial cell factory for enzyme production. The development of tools that allow genetic manipulation is crucial for further strain improvement and the functional characterization of its genes. In this context, the CRISPR/Cas9 system represents an excellent option for genome editing due to its high efficiency and versatility. To establish CRISPR/Cas9 genome editing in *P. subrubescens*, first a method for protoplast generation and transformation was developed, using hygromycin as selection marker. Then the CRISPR/Cas9 system was established in *P. subrubescens* by successfully deleting the *ku70* gene, which is involved in the non-homologous end joining DNA repair mechanism. Phenotypic characterization of the mutants showed that *ku70* mutation did not affect *P. subrubescens* growth at optimal temperature and Δ*ku70* strains showed similar protein production pattern to the wild type.

1. Introduction

Filamentous fungi have great potential in multiple industrial applications due to their ability to produce a broad source of proteins, enzymes, organic acids and secondary metabolites [1,2]. However, fungi can also produce other metabolites such as mycotoxins, which have undesired effects on plant, animal and human health [3,4].

Fungal enzymes especially from the genera *Aspergillus*, *Trichoderma* and *Penicillium* have been commercially used for many applications [5–7]. *Aspergillus niger* is one of the most widely used fungi for the production of primary metabolites and extracellular enzymes. The latter include a wide range of Carbohydrate-Active enZymes (CAZymes) involved in plant biomass degradation for their application in several industrial fields, such as food and feed, pulp and paper, textile, detergents, and biofuels and biochemicals [8–10]. In contrast, the potential of *Penicillium* species for the production of commercially interesting compounds has been less studied and exploited. Some relevant examples include the utilization of *Penicillium oxalicum* as cell factory for the production of many industrially relevant enzymes, including cellulases and xylanases [11,12], and *Penicillium rubens,* from which the antibiotic penicillin was originally isolated [13]. However, in the last decade, *Penicillium* species got wider recognition as potential cell factories for enzyme production [12].

Penicillium subrubescens has been reported to be a promising alternative for *A. niger* in plant biomass degrading-enzyme production [14]. It shows a very good ability to saccharify complex plant biomass, and its genome sequence reveals an enriched content of specific CAZyme families compared to other filamentous fungi, including *A. niger* [15]. Additionally, *P. subrubescens* has been demonstrated to efficiently degrade inulin [16], a storage carbohydrate present in plants that can be used as raw material for the production of high fructose syrup, as prebiotic and bifidogenic agent, and as raw material for bioethanol production [17]. All this makes *P. subrubescens* a potential industrial cell factory for enzyme production. To achieve such a status, it is crucial that efficient methods for strain development are available.

Given the broad applicability of fungal enzymes in the industry, increasing enzyme production as well as avoiding undesired enzymatic activities have become the main objectives over the last decades. Usually wild-type strains do not naturally produce the protein amounts required at industrial levels, or the enzyme cocktails they produce are not the optimal or desired ones. For this reason, a number of genetic engineering approaches have been developed in order to overcome these problems [18,19].

The increasing availability of genome sequences has greatly expanded the toolkit for fungal genetic engineering. In this context, The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-

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associated RNA-guided DNA endonucleases (Cas) technology, which originates from archaeal and bacterial immune systems, has become a newly applied technology in filamentous fungi for gene editing because of its high efficiency, versatility and straightforward design [20,21]. The CRISPR/Cas9 technology has enabled the simple and cost-efficient genetic manipulation of some model and non-model filamentous fungi, including basidiomycete species [22–24].

In the present study, we developed an efficient genetic transformation method for the filamentous ascomycete *P. subrubescens* and demonstrate the establishment of CRISPR/Cas9 genome editing for this fungus. As case study, we successfully targeted the *ku70* gene involved in the Non-Homologous End Joining (NHEJ) DNA repair mechanism [25], in order to generate *P. subrubescens* NHEJ-deficient strains with improved homologous recombination frequency that could be used as a parental strain for subsequent genetic modifications with efficient site-specific recombination.

2. Material and methods

2.1. Strains, media and growth conditions

Escherichia coli DH5α was used for plasmid propagation, and was grown in Luria-Bertani (LB) medium supplemented with 50 μg/mL ampicillin (Sigma Aldrich).

P. subrubescens CBS132785 was used as parental strain. The generated Δ*ku70* mutant was deposited at the culture collection of Westerdijk Fungal Biodiversity Institute under the accession number CBS145939 (subsequently referred to as Δ*ku70*_1). For strain propagation, *P. subrubescens* wild type and Δ*ku70* strains were routinely cultured on Malt Extract Agar (MEA) plates for 5–7 days at 30°C. Conidia were subsequently harvested, dispersed in ACES buffer, and the concentration was adjusted using a haemocytometer.

For the temperature sensitivity tests, 2 μL of ACES buffer containing 10^3 conidia of the different *P. subrubescens* strains were inoculated on either 1% D-glucose Minimal Medium (MM) [26] or Potato Dextrose Agar (PDA) plates. Plates were incubated at 25, 30, and 34°C for 10 days, and growth diameter was measured daily.

Phenotypic analysis of the deletion strains was performed in duplicates on MM plates containing 1.5% (w/v) agarose with 25 mM D-glucose, D-fructose, D-galactose, D-mannose, D-ribose, D-xylose, L-arabinose, L-rhamnose, D-galacturonic acid, D-glucuronic acid, cellobiose, maltose, lactose, raffinose, or sucrose; 1% arabinogalactan, beechwood xylan, arabic gum, guar gum, soluble starch, apple pectin (pH 6), citrus pectin (pH 6), inulin, lignin, casein or cellulose; and 3% wheat bran (WB), sugar beet pulp (SBP), citrus pulp, soybean hulls, rice bran, cotton seed pulp, or alfalfa meal. Plates were inoculated with 2 μL of ACES buffer containing 10^3 spores and incubated at 30°C for up to 14 days. Growth was assessed by visual inspection.

2.2. DNA construction

For the design of the 20 bp protospacer for the single guide RNA sequence (sgRNA), the Geneious 11.04.4 software was used (https://www.geneious.com). The sgRNA sequence (Table 1) with no off-target and the highest on-target activity was predicted based on the experimentally determined predictive model [27]. The sgRNA was then synthesized (IDT, Integrated DNA Technology) and used to delete the *ku70* gene (gene ID: PENSUB_5344) in *P. subrubescens* CBS132785.

The construction of the CRISPR/Cas9 vector pFC332 with the specific sgRNA to target the *ku70* gene was performed as previously described [28] (Fig. 1). Briefly, pFC332 contains the hygromycin resistance gene (*hph)* under the control of the *A. nidulans trpC* promoter, the Cas9 nuclease-encoding gene, which was codon-optimized for *Aspergillus,* under the control of the *A. nidulans tef1* promoter, and the AMA1 sequence to support self-replication of the plasmid (Fig. 1A). Plasmid pFC334 (Fig. 1B) was used as template to generate the two PCR fragments for the construction of the sgRNA (Fig. 1C). All primers used to generate the two sgRNAs fragments are listed in Table 1. PCR amplifications were performed with Phusion U Hot Start DNA Polymerase (Thermo Fisher Scientific). After digestion of the pFC332 backbone with the restriction enzymes PacI/Nt.BbvCI (Biolabs), the construction of the CRISPR/Cas9_sgRNA plasmid (Fig. 1D) was generated inserting the two PCR fragments by USER cloning in one single step.

The rescue template (RT) was obtained through Fusion PCR amplification using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). First, two PCR fragments were generated by amplifying approx. 1500 bp upstream and downstream of the *ku70* gene. Finally, these two fragments were fused together in a second nested PCR event obtaining ~ 2000 bp RT with approx. 1000 bp homology arms to both upstream and downstream regions of the *ku70* gene, and was subsequently purified (Wizard® SV Gel and PCR clean-Up System, Promega) and co-transformed with the CRISPR/Cas9 plasmid. All the primers used to generate the RT are listed in Table 1.

2.3. Protoplast generation, fungal transformation and confirmation of the mutant strains

Protoplasts from *P. subrubescens* CBS132785 and fungal transformation were performed as previously described [29] with some modifications [30]. A final concentration of 4×10^6 conidia/mL spores of *P. subrubescens* was inoculated in a 1 L Erlenmeyer flask containing 250 mL of 2% D-glucose Transformation Medium (TM). After 24 h at 30°C and 250 rpm, the pre-culture was vacuum filtered, washed with 0.6 M $MgSO_4$, and dried. The mycelium was subsequently dissolved in 50 mL PS buffer (0.2 M sodium phosphate buffer, 0.8 M L-sorbitol, pH 6) containing VinoTaste® Pro lysing enzyme (Novozymes) (0.5 *g* enzyme / g of mycelium). The mix was incubated in a rotary shaker at 30 °C, 100 rpm for 3.5 h. When protoplasts were abundant, the suspension was filtered through sterile Miracloth paper, and centrifuged for 10 min at 4 °C and 1800 × *g*. Protoplasts were resuspended in 10 mL of solution B (1 M L-sorbitol, 50 mM $CaCl_2$, 10 mM Tris − HCl, pH 7) and washed once. Finally, protoplasts were resuspended in solution B to a final concentration of 2×10^7 protoplast/mL. For transformation, 200 μL of fresh protoplasts (2×10^7 protoplast/mL) were mixed with 50 μL of solution C (25% PEG 6000, 50 mM $CaCl_2$, 10 mM Tris − HCl, pH 7.5), and 10 μL DNA containing 3 μg of the CRISPR-Cas9 plasmid and 6 μg of RT. The mixture was incubated on ice for 20 min. After incubation, 2 mL of solution C were added and the solution was incubated for 5 min at room temperature. Finally, 2 mL of solution B were added to the protoplast suspension and it was spread over two hygromycin-containing plates (200 μg/mL) of MM supplemented with 0.95 M sucrose pH 6 and 1 M L-sorbitol. Plates were incubated at 30°C until colonies were observed (7–10 days). Transformants were purified by one single colony strake to selective plates and four consecutive single colony streaks to non-selective plates in order to make them lose the plasmid. Genomic DNA of the transformants was isolated using the Wizard® Genomic DNA Purification Kit (Promega) and mutants were confirmed by PCR amplification using the primer pairs indicated in Table 1 and by Sanger sequencing (Fig. 2).

2.4. Protein production analysis

For protein production and comparison analyses, 10^6 spores/mL of *P. subrubescens* strains were inoculated in 250 mL Erlenmeyer flasks containing 50 mL MM supplemented with either 1% WB, 1% SBP, or 1% D-fructose. All liquid cultures were incubated in a rotary shaker at 30°C and 250 rpm for up to 6 days. Experiments were carried out in biological duplicates. Supernatant samples were taken after 3 and 6 days of incubation and were centrifuged for 10 min at 13,500 × *g*. Ten μL of each sample were analyzed by SDS-PAGE using SDS-12% polyacrylamide gels calibrated with PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) and silver stained.

Table 1
Primers used in this study.

Primer ID	Use[a]	Sequence (5'→ 3')	Purpose[b]
pFC1	F	GGGTTTAAUGCGTAAGCTCCCTAATTGGC	sgRNA
pFC2	R	GGTCTTAAUGAGCCAAGAGCGGATTCCTC	sgRNA
PsKu_5R	R	AGCTTACUCGTTTCGTCCTCACGGACTCATCAGGGACAACGGTGATGTCTGCTCAAGCG	sgRNA
PsKu_3F	F	AGTAAGCUCGTC**GGACAATTGCAGCTTCCAAA**GTTTTAGAGCTAGAAATAGCAAGTTAAA	sgRNA
Ps-ku70_5F	F	GCAGCACAACCTCCTTTCG	RT
Ps-ku70_5R	R	CGATAGCGAATCCTAGCAGTACCAGCCTTTGCGTCATCG	RT
Ps-ku70_3F	F	ACTGCTAGGATTCGCTATCGTCATGTCAGCAGTGGGTGCG	RT
Ps-ku70_3R	R	GGAAGTGCCTCATCCTCCAT	RT, cs
Ps-ku70NEST_5F	F	TCGCTTCTCCTCAGGGTAGG	RT, cs
Ps-ku70NEST_3R	R	GGTCAATTTCTGGCACGGAG	RT
Psksc_5F	F	CTGTGTGACTGGAGGTACAGC	seq
Psksc_3R	R	ATGACTTCTCTCATGGCGTCG	seq

The sgRNA sequence is highlighted in bold.
[a] F: forward, R: reverse.
[b] sgRNA: single guide RNA; RT: Rescue Template; cs: colony screening; seq: Sanger sequencing.

3. Results

3.1. CRISPR/Cas9 system was successfully applied to generate Δku70 mutants in P. subrubescens

After the optimization of the protoplast generation and transformation protocols (see material and methods), and the determination of a proper hygromycin concentration for transformants selection (200 μg/mL), the CRISPR/Cas9 genome editing system was tested in *P. subrubescens*. The protocol used to obtain Δ*ku70* mutants includes the co-transformation of the single, self-replicating CRISPR/Cas9 plasmid pFC332 (Fig. 1) and a linear fragment of DNA known as rescue template (RT). *In vivo,* Cas9 is directed to the specific DNA locus by the sgRNA, where it makes a double-strand break (DSB) [31]. On the other hand, the RT comprises a linear fragment of DNA obtained by PCR amplification and fusion of the 5′ upstream and 3′ downstream flanking regions of the *ku70* gene. Since previous studies in other filamentous fungi including *Penicillium chrysogenum* [32] and *T. reesei* [33] showed that the DSBs generated by Cas9 could be efficiently repaired by 1 kb complementary DNA arms, this size was used to obtain the RTs that would repair the damage in *P. subrubescens* by homologous recombination.

After 10 days post-transformation, hygromycin-resistant colonies were transferred, up to four times, to non-selective 1% D-glucose MM plates in order to cause the loss of the CRISPR/Cas9 plasmid containing the hygromycin resistance to ensure the recycling of the system. Finally, the transformants were molecularly confirmed using specific primer combinations designed outside or inside the replacement construction to discriminate positive deletants from parental strain (Fig. 2). The positive Δ*ku70* deletion mutants were inoculated on hygromycin-containing plates to confirm the absence of resistance (data not shown). Two out of twenty-two hygromycin-resistant transformants were positive *P. subrubescens* Δ*ku70* mutants as confirmed by PCR (Fig. 2B). Both showed clean *ku70* deletions, which were additionally confirmed by Sanger sequencing (Fig. 2C) and phenotypically analyzed (see below).

In order to investigate the possible toxic effect of the Cas9 endonuclease towards *P. subrubescens,* two additional transformations were performed to compare the transformation efficiency (number of hygromycin-resistant colonies after transformation) of the *cas9*-containing plasmid pFC332, and the plasmid pPAN7-1 [34] used as an empty vector control without either the *cas9* or the sgRNA expression cassettes. Our results showed that the transformation efficiency obtained after these two transformation events is comparable, indicating that the Cas9 endonuclease has no negative effect towards *P. subrubescens* (Supplementary Fig. 1).

3.2. Growth analysis of P. subrubescens Δku70 mutants at different temperatures

To phenotypically characterize *P. subrubescens* Δ*ku70* strains, their sensitivity to different temperatures was tested. Mutants were grown at 25, 30 and 34 °C for ten days in two different culture media: MM + 1% D-glucose and PDA (Fig. 3). The results showed no growth differences between the mutants and wild-type strain at 30 °C, which is the optimal growth temperature for this fungus, on both MM and PDA. At 34 °C, the growth of all strains was similar on MM, but mutants showed a small growth reduction on PDA. At 25 °C, the growth of all strains was slower and the mutants showed reduced growth on both culture media (Fig. 3). Finally, a slight decrease in conidia production was observed in the Δ*ku70* mutants in MM at all temperatures, but not in PDA (Fig. 3A).

3.3. Phenotypic analysis of P. subrubescens Δku70 mutants

Using the temperature at which optimal growth was observed (30 °C), the Δ*ku70* mutants were grown on MM plates supplemented with a wide variety of carbon sources, including simple and complex sugars and other crude substrates, and the growth was compared to that of the parental strain (Fig. 4). Results showed that *ku70* deletion does not affect growth, since phenotypes of the Δ*ku70* strains were not significantly different from those of *P. subrubescens* wild type after 7 days of growth in all carbon sources tested (Fig. 4).

3.4. Protein production analysis

P. subrubescens is an ascomycete fungus with high potential for enzyme production, similarly to the industrially relevant *A. niger* [14]. In order to study whether the deletion of the *ku70* gene affected protein production, Δ*ku70* mutants were grown in liquid MM supplemented with different carbon sources: D-fructose as non-inducing protein production condition, and wheat bran (WB) or sugar beet pulp (SBP) as inducing conditions. After 3 and 6 days of growth, the extracellular protein profile of the Δ*ku70* mutants was compared to that of the parental strain (Fig. 5). Lower amounts of proteins were visible in the fructose culture samples (Fig. 5A), while protein production was much more abundant under inducing conditions (Fig. 5B–C). In all cases the Δ*ku70* mutants showed a very similar protein pattern in the three different culture conditions after 3 and 6 days post-inoculation (Fig. 5), although some differences can be noticed. Under non-inducing conditions, no difference in protein production was observed between the parental and the mutant strains after 3 days of growth. However, ~60 kDa proteins are more abundant in the parental strain after 6 days (Fig. 5A). Under inducing conditions, an increased production of ~35 kDa proteins can be seen in the mutant strains when grown on WB

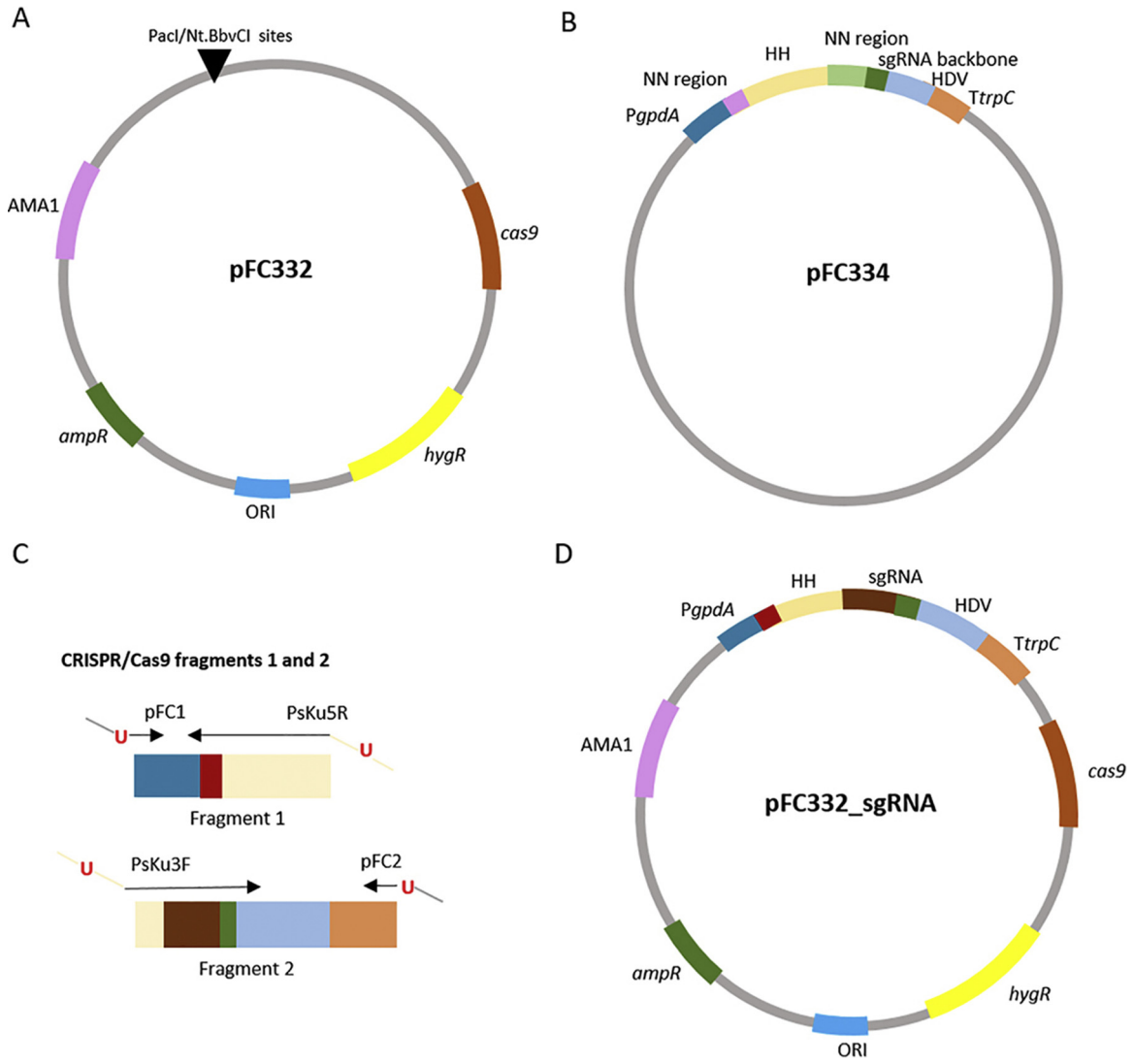

Fig. 1. Schematic representation of the CRISPR/Cas9 vectors for *ku70* gene mutagenesis in *P. subrubescens*.
A) The pFC332 plasmid contains the *cas9* gene under the control of the *tef1* promoter from *A. nidulans* (orange), the hygromycin resistance gene (*hph*) under the control of the *trpC* promoter from *A. nidulans* (*hygR*, yellow), a β-lactamase gene for ampicillin selection (*ampR*, green), an *E. coli* origin of replication (ORI, blue), the AMA1 sequence to support self-replication of the plasmid (pink), and a *Pca*I and Nt.*Bbv*CI restriction enzyme cutting site to facilitate the insertion of the sgRNA gene. B) Plasmid pFC334 used as template to generate two PCR fragments for the construction of the sgRNA to delete the *ku70* gene. It includes two variable sequence regions (NN regions). The first NN region (light green) is replaced by the 20 bp protospacer, while the second NN region (pink) contains 6 bp that is replaced by 6 bp corresponding to the inverted sequence of the 5′-end of the protospacer to complete the hammerhead ribozyme (HH) cleavage site. Both protospacer and the inverted 6 bp sequences at the NN regions are introduced *via* tails added to the ends of the two corresponding primers that define the down- and upstream ends of the two PCR fragments, respectively. C) PCR fragments from pFC334. The primers used to generate these fragments are represented as arrows. Fragment 1 contains the *A. nidulans gpdA* promoter (dark blue), the major part of the hammerhead ribozyme (HH) coding gene (beige), and the 6 pb inverted sequence from the 5′-end of the protospacer (red). Fragment 2 contains the last part of the HH coding gene (beige), the sgRNA including the protospacer (brown) and the sgRNA backbone (dark green), the hepatitis delta virus (HDV) ribozyme coding gene (light blue), and the *trpC* terminator (orange). Note that the primers upstream and downstream of the fragments 1 and 2 contain a tail with a uracil base (red) that is necessary for the USER cloning step. D) Final CRISPR/Cas9 plasmid pFC332 containing the sgRNA sequence ready to be transformed in *P. subsubescens*. Color codes are as in A, B and C. DNA elements are not drawn to scale. For more detailed information see [28].

after 3 and 6 days post-inoculation (Fig. 5B), whereas ~20 kDa proteins are more abundant in the parental strain on both WB and SBP (Fig. 5B–C).

4. Discussion

The establishment of the CRISPR/Cas9 system for genome editing has been previously reported in several filamentous fungal species including *Alternaria alternata*, *A. niger*, *Aspergillus nidulans*, *Aspergillus acuelatus*, *T. reesei*, *Magnaporthe oryzae*, *Ustilago maydis* and *P. chrysogenum* [28,32,33,35,36]. In this study, we demonstrate the establishment of the CRISPR/Cas9 system in the filamentous fungus *P. subrubescens,* which has high potential as an industrial enzyme cell factory. Establishment of new industrial cell factories is often hampered by a lengthy process of strain improvement, but the efficiency of CRISPR/Cas9 facilitates a fast development of novel candidate cell factories. The CRISPR/Cas9 approach used in this study has some specific advantages, such as the use of a self-replicating plasmid that does not integrate into the genome, allowing the isolation of cured strains and recycling of the transformation system.

As case study and to obtain a starting strain for future genome editing, the *ku70* gene was deleted in *P. subrubescens.* The Ku70/Ku80 protein complex is involved in the non-homologous end joining (NHEJ) DNA repair pathway and has relevant functions in the maintenance of

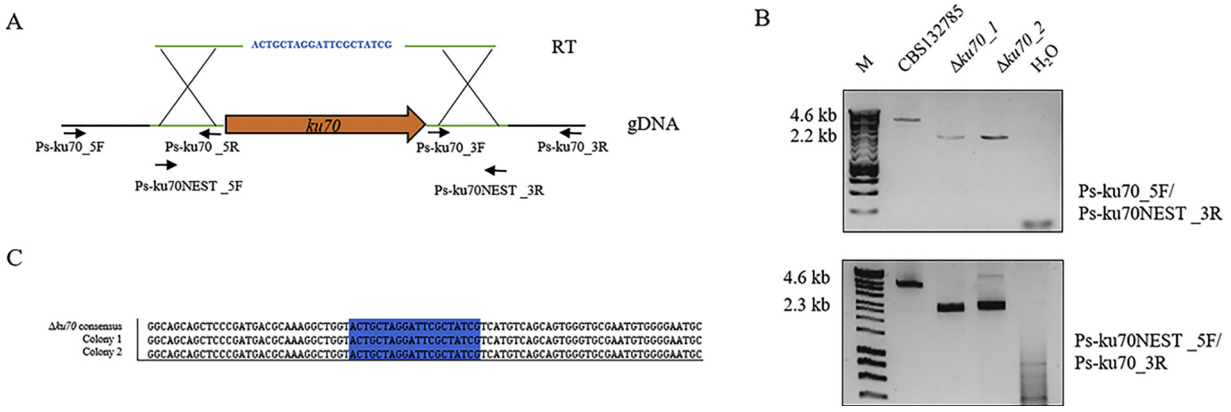

Fig. 2. Molecular characterization of *P. subrubescens* Δ*ku70* stains.
A) Schematic representation of the genomic DNA (gDNA) reparation by homologous recombination after Cas9 double strand break inside the *ku70* gene. Orange arrow represents *P. subrubescens ku70* gene. The 1 kb complementary arms to the upstream and downstream regions of the *ku70* gene are represented in green. To generate the rescue template (RT), a randomly chosen 20-nucleotide sequence was introduced for complementarity during the fusion PCR of the two 1 kb homology arms (blue). Black arrows represent the primers used for RT generation and transformant confirmation by PCR. B) PCR confirmation of the *P. subrubescens* Δ*ku70* strains. Lower PCR bands (~2.2–2.3 kb) correspond to the deletants whereas the upper bands (~4.6 kb) correspond to the parental strain. The primer pairs used are as indicated in the figure. C) Δ*ku70* strains confirmation by Sanger sequencing. The barcode is represented in blue.

genome integrity in eukaryotic organisms. However, it has been shown that the disruption of *ku70/ku80* genes does not significantly affects genome stability in filamentous fungi but rather improves the efficiency of the homologous directed repair pathway favoring homologous recombination [37–39]. Moreover, the NHEJ is the dominant mechanism to repair double strand DNA breaks in filamentous fungi [40,41]. In this context, previous studies reported that mutants in *ku70* gene decreased NHEJ and increased the homologous recombination pathway to nearly 100% in many fungal species [33,38,42,43]. Identification of Δ*ku70* mutants confirmed the functionality of the CRISPR/Cas9 system in *P. subrubescens*. Nevertheless, although the mutation rate obtained (2 positive mutants out of 22 analyzed) is comparable to that obtained for *A. alternata* [36], the efficiency was not as high as reported in other studies where the same CRISPR/Cas9 system was applied [28,32,33,44]. Many factors are involved in the CRISPR/Cas9 gene editing efficiency and could have influenced our results, such as the expression efficiency of sgRNAs, codon usage of the Cas9 endonuclease [28], or the 20-nucleotide protospacer design [30,45]. These factors

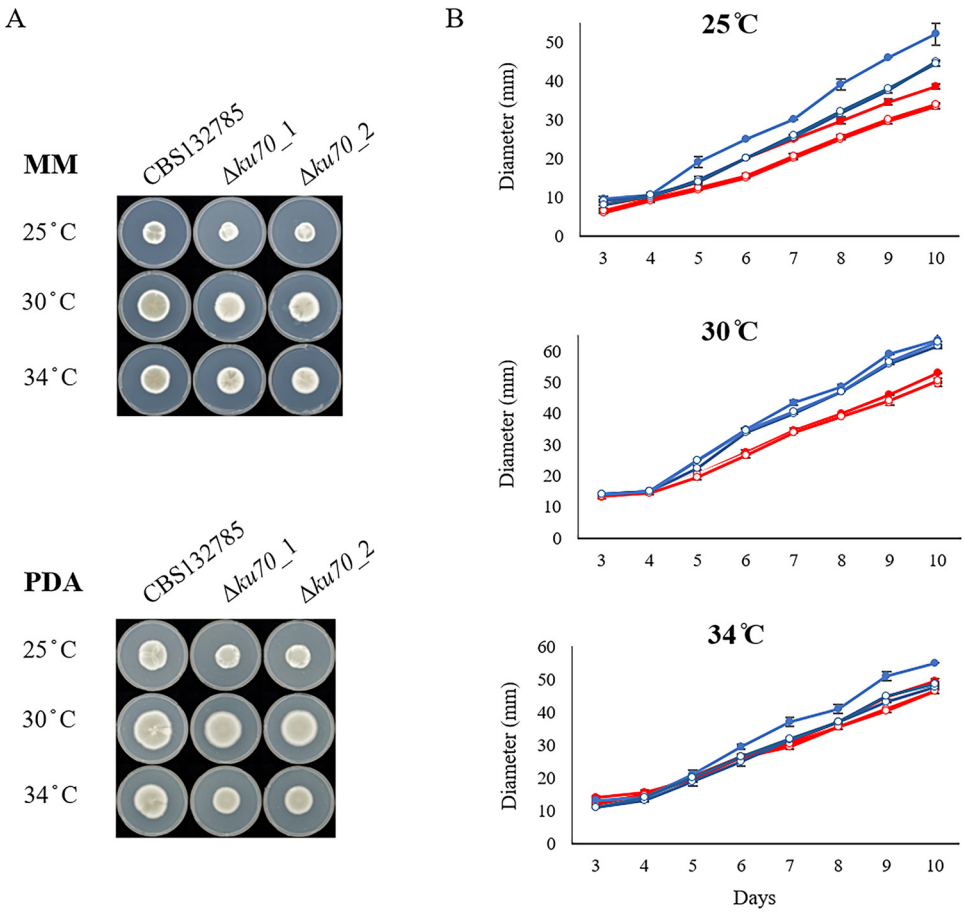

Fig. 3. Phenotypic characterization of *P. subrubescens* Δ*ku70* strains at different temperatures.
A) Growth morphology of the parental CBS132785 and Δ*ku70* strains after 8 days of growth at 25, 30 or 34°C on MM + 1% D-glucose or PDA plates, as indicated. B) Colony diameter measurement from 3 to 10 days of growth at 25, 30 or 34°C on MM + 1% D-glucose (red lines) or PDA plates (blue lines) of the parental strain (filled circles) and the mutant strains (white circles). Data are mean and standard deviation (SD) values of two technical replicates.

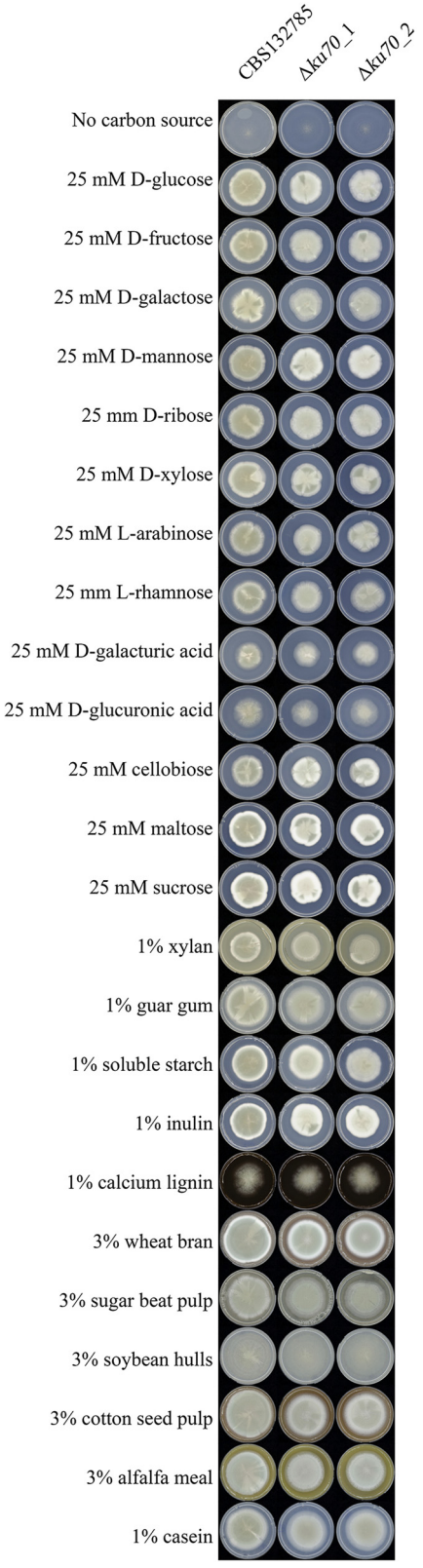

Fig. 4. Phenotypic analysis of *P. subrubescens* Δ*ku70* strains on different carbon sources.
Two independent mutant strains, Δ*ku70*_1 and Δ*ku70*_2, were used and growth was compared to that of the parental strain. Plates were inoculated with 10^3 conidia and incubated at 30°C. Pictures were taken after 7 days of growth.

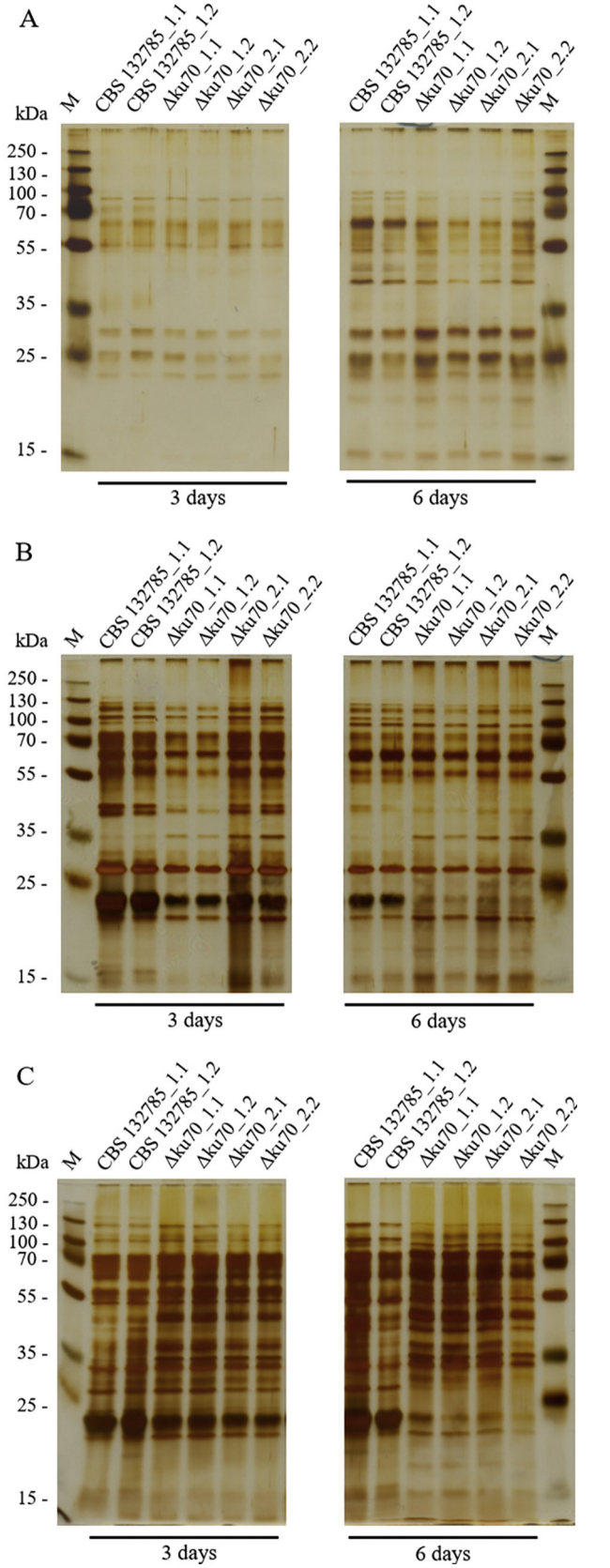

Fig. 5. Protein production analysis of *P. subrubescens* Δ*ku70* strains.
SDS-PAGE analysis of the supernatants of *P. subrubescens* wild-type, Δ*ku70*_1 and Δ*ku70*_2 strains after 3 and 6 days of growth in MM + 1% D-fructose (A), 1% WB (B), and 1% SBP (C). Ten µL of each supernatant were loaded per well. Two biological replicates are shown per strain. M: PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific).

need to be taken into account to improve genome editing rates when using CRISPR/Cas9 and will be addressed in the future to increase the efficiency of the system in *P. subrubescens*. Toxicity of Cas9 endonuclease could also affect genome editing efficiency. However, this does not seem to be the case of *P. subrubescens* (Supplementary Fig. 1). Cas9 endonuclease has been successfully applied in *P. chrysogenum* [32] and other closely related species such as *Aspergillus fumigatus*, *Aspergillus oryzae,* and *A. niger* [41,45,46], and no toxicity has been reported in Eurotiomycetes to date.

Once *P. subrubescens* Δ*ku70* mutants were obtained, they were genotypic and phenotypically characterized. Previous studies reported phenotypic differences between Δ*ku70* and wild-type strains in some filamentous fungal species. For example, it was reported that the inactivation of the *ku70* gene decreased conidia formation and pigment production in the fungus *A. alternata* [47]. In addition, increased temperature sensitivity was reported in *Penicillium digitatum* Δ*ku70* strains [48]. In contrast, *A. niger* and *P. chrysogenum* Δ*ku70* strains were reported to be similar to their parental strains regarding axenic growth and conidia production, although they exhibited higher sensitivity to UV radiation and osmotic stress, respectively [49,50]. These observations confirm important roles of the Ku complex in fungal development, maintenance of genomic integrity and response to stress, which may differ depending on the fungal species under study. Our results showed that *ku70* deletion did not significantly affect *P. subrubescens* growth rate or protein production patterns at optimal temperature in different culture conditions, even though Δ*ku70* strains showed a slight reduction in colony diameter at sub-optimal temperatures except for MM at 34 °C. However, none of these differences were substantial enough to likely hamper application of this strain in biotechnology.

5. Conclusions

We have demonstrated the implementation of the CRISPR/Cas9 system in the filamentous fungus *P. subrubescens* and effective protoplast generation and transformation protocols were optimized. As case study, Δ*ku70* strains were generated with this system, and the mutants showed no significant phenotypic differences with the wild type at optimal temperature, allowing the use of these mutants as parental strains for subsequent transformation events. With this work, we expand the repertoire of fungi where genetic engineering is possible and thus contribute to accelerate the study and exploitation of the potential of *P. subrubescens* as cell factory at biotechnological and industrial level. This study also exemplifies the new possibilities genome editing using CRISPR/Cas9 or similar methodology has to offer for industrial biotechnology.

Conflicts of interest

None.

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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.enzmictec.2019.109463.

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