Cloning and Expression of an Active Aspartic Proteinase Gene From *Aspergillus Oryzae* DRDFS13 in *Pichia Pastoris*

Jermen Mamo

Debre Berhan University <https://orcid.org/0000-0003-0776-3310>

Konstantina Kostadinovska

Downstream Processing Laboratory, Department of Life Sciences and Chemistry, Jacobs University, Bremen, Germany

Martin Kangwa

Downstream Processing Laboratory, Department of Life Sciences and Chemistry, Jacobs University, Bremen, Germany

Marcelo Fernandez-Lahore (✉ m.fernandez-lahore@jacobs-university.de)

Department of Life Science and Chemistry, Jacobs University

Fassil Assefa

Department of Microbial, Cellular and Molecular biology

Research

Keywords: *Aspergillus oryzae*, Milk-clotting activity, Recombinant protein, Recombinant yeast

Posted Date: November 5th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-101507/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Pichia pastoris is a yeast widely used in expressing recombinant proteins from eukaryotic organisms. In the present study, the total RNA was extracted from a eukaryotic fungus; *Aspergillus oryzae* DRDFS13 and reverse transcribed into cDNA using specific primers.

The gene for aspartic protease was amplified and sequenced and then cloned into pGAPZαA for further expression in *P. pastoris*. The recombinant yeast (*P. pastoris* X-33Ap) was cultivated in YPD media at pH 5 and 7 for 6 days and the production of recombinant proteins was checked by total protein determination, milk-clotting activity assay, and SDS-PAGE analysis.

Results

The gene sequence results showed 98% similarity with aspartic protease gene from *A. oryzae* RIB40. The aspartic protease gene cloned into pGAPZαA (later pMKAP) was successfully expressed in *P. pastoris* as an active extracellular protease with the highest MCA (190.47 MCU/mL) of secreted enzyme from the recombinant yeast was obtained at pH 5 and 6 days of incubation time. The major protein expressed by the recombinant *P. Pastoris* X-33 AP has a molecular mass between 32 and 46 kDa. When analyzed for clotting activity, the protein was able to clot skim-milk in 2 min. The clotting activity was found to be 190.47 U/mL.

Conclusion

Thus, the milk-clotting protease extracted from the recombinant yeast in the present study could be a suitable candidate for cheese production. However, further study of the recombinant proteins need to be carried out and its application in cheese production by analyzing the organoleptic and chemical properties of the cheese produced.

1. Background

Acid proteases are widely utilized in the food, beverage, and pharmaceutical industries. The major application of acid proteases is in cheese production in the dairy industry. Several species of fungi such as *Aspergillus*, *Candida*, *Rhizomucor* and *Phanerochaete chrysosporium* are reported to yield high amounts of aspartic protease enzymes while *Aspergillus* spp is the major producers for they possess several encoding genes such as pepA (Gomi *et al.*, 1993; Nair & Jayachandran, 2019).

Aspartic protease genes from fungi may be expressed in yeast for large scale fermentation as it is reported that yeast is good expression hosts for genes of fungal origin (Sun *et al.*, 2018; Yegin and Fernandez-Lahore, 2013). The authors reported that the methylotrophic yeasts such as *Pichia pastoris* are widely used as expression platforms for recombinant proteins for basic research and industrial applications.

The methylotrophic yeast *Pichia pastoris*, currently reclassified as *Komagataella pastoris*, has become a substantial workhorse for biotechnology, especially for heterologous protein production (Ahmad et al., 2014). *P. pastoris* have many advantages in yielding a high-level expression of recombinant proteins, protein processing and are characterized by post-translational modifications (Kangwa et al., 2018; Luo et al., 2016). The post-translation modification associated with higher eukaryotes such as processing of signal sequence, folding, disulfide bridge formation, certain types of lipid addition and *O*- and *N*-linked glycosylation (Cereghino and Cregg 2000). It can also be cultivated on inexpensive media with low-level proteins and has been accepted as a safe and effective expression system by the U.S. Food and Drug Administration (FDA) (Luo et al., 2016). *P. pastoris* has been shown great achievement in the large-scale production of recombinant protein (Cregg et al. 2000).

The expression of the aspartic protease in *P. pastoris* is achieved by cloning the protease gene into the expression vector pGAPZ α -A. pGAPZ α A is chosen as an expression vector since it is designated for high-level constitutive expression in *P. pastoris* (Cereghino and Cregg 2000). The pGAPZ α A was created when the methanol-regulated AOX1 promoter was replaced with a constitutive, glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter in the vector. The advantage of using the GAP promoter is that there is no need to shift cultures from one carbon source to another as methanol is not required for induction of the enzyme. It is established that genetic manipulation in filamentous fungi is more complex than in yeast and bacteria and so necessitates expression of the desired gene from filamentous fungi into a suitable host. For the last two years, we collected and screened several bacteria and fungi for their aspartic protease activities in our laboratory, of which, some of the local filamentous fungi showed potential for aspartic protease activity in a pilot-scale study.

The aim of the present work is to study the expression of an aspartic protease gene from the local *A. oryzae* DRDFS13 strain into *P. pastoris*. The gene was cloned in pGAPZ α A and inserted into *E. coli* K-12 for plasmid amplification and transformed in *P. pastoris* for protein expression. The milk-clotting activity of the protein was investigated for its potential in cheese manufacturing.

2. Materials And Methods

2.1. Fungal and bacterial strain

The microorganism used as the source of the gene encoding aspartic protease enzyme was *Aspergillus oryzae* DRDFS13. The fungal strain was grown at 30 °C for 3 days in a liquid broth (Potato Dextrose Broth).

E. coli K-12 ER2738 was used to amplify the plasmids carrying the cloned gene. *E. coli* strains were grown overnight in Luria-Bertani medium (10 g L⁻¹, tryptone, 5 g L⁻¹ yeast extract, 5 g L⁻¹ NaCl) at 37 °C, 220 rpm. *P. pastoris* X-33 was grown in YPD medium (10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, 20 g L⁻¹ glucose) at 30 °C for 3 days with shaking at 250 rpm (Kangwa et al. 2018).

2.2. cDNA synthesis

First, total RNAs were extracted from the mycelia of *Aspergillus oryzae* DRDFS13 using a NucleoSpin® RNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's standard protocol (Yegin and Fernandez-Lahore, 2013). Then, the first-strand cDNA was synthesized from RNA using the ProtoScript® II First Strand cDNA Synthesis Kit (#E6560S, New England Bio Labs Inc, Brünigstraße 50, 65929 Frankfurt am Main) according to the manufacturer's protocol. The reaction components were mixed and incubated at 42 °C for 1 h, followed by heat inactivation at 80 °C for 5 min. Products were then stored at -20 °C for further amplification of the aspartic protease gene (Antonio *et al.*, 2013).

2.3. Aspartic protease gene amplification and sequencing Amplification of the aspartic protease gene was done using forward primers APJM_Fw01 5'-CCT CGA GCA TGG TTA TCT TGA GCA AAG TCG C-3' and reverse primer APJM_Rw01 5'-GCG GCC GCC AAG CCT GGG CGG CGA AGC CGA G-3', other PCR components were: 10 µl of 5x Phusion High Fidelity buffer, 1µl of 10 mM dNTPs solution mix, 0.5 µl of 2,000U Phusion High fidelity Polymerase, all purchased from New England Bio Labs Inc, Frankfurt am Main, 2.5 µl of 10 mM primer (forward and reverse) synthesized by Eurofins genomics, 2 µl of plasmid DNA (50 ng/µl), and sterile distilled water was added to the final volume of 50 µl. From the amplified DNA, 15 µl of sample was run on a 1% agarose gel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with 1X TPE buffer [stock concentration, 10X: 1 M Tris base, 20 mM EDTA, 225 mM phosphoric acid all products of Applichem GmbH, Darmstadt, Germany] at 90 V, maximum Amps for 55 minutes. The gel was stained in ethidium bromide solution and viewed using a Gel documentation system. The remaining PCR product (gene) was purified using a Nucleospin Plasmid Kit according to the manufacturer's protocol as described by Macherey-Nagel (2012). The resulting samples were eluted in 30 µl of elution buffer; concentrations were measured using a Nanodrop-2000 Spectrophotometer. Samples were then sent to Eurofins Genomics for sequencing. The results were analyzed in comparison to the protein amino acid sequence of *Aspergillus oryzae* RIB140 Aspartic protease sequences (Antonio *et al.*, 2013).

2.4. Cloning and expression of aspartic proteinase gene in *pastoris* The aspartic protease gene was inserted into pGAPZαA using the restriction enzymes XhoI and NotI (both from New England Bio Labs Inc) thereby producing an expression vector pMK-AP with the 6xHis tag at the C-terminal and kanamycin for selection in bacteria, and while zeocin as for selection in yeast (Yegin and Fernandez-Lahore, 2013). Then the pMK-AP vector was transformed to *E.coli* K-12 ER2738 competent cells by electroporation at 1.8 kV for 5 milliseconds (Yegin and Fernandez-Lahore, 2013).

The tubes carrying competent cells were incubated at 37 °C with shaking for 60 min. Then two types of samples (concentrated and un-concentrated) were prepared for plating. For the un-concentrated samples, 100 µl was taken from the Eppendorf tube directly after incubation. While the preparation of the concentrated samples involved centrifugation for a minute (11000 rpm at 4 °C). Then 700 µl of the supernatant was discarded and the pellet was re-suspended in the remaining 200 µl. The cells were cultivated on LB agar plates supplemented with 25 µg/mL tetracycline and 25 µg/mL Kanamycin final concentrations (Carl Roth GmbH, Karlsruhe, Germany) and incubated overnight at 37 °C. Positive colonies carrying the coding sequence of aspartic protease gene were identified by colony PCR by lysing a colony

in 10 μ L sterile water heated to 100 °C for 10 minutes. One microliter of the lysed colony was further used in colony PCR using previously used primers and sequencing (Yegin and Fernandez-Lahore, 2013).

Plasmids carrying the AP coding sequence were extracted from positive colonies using a NucleoSpin® Plasmid Isolating Kit and further digested with *AvrII* restriction enzyme and inserted into *P. pastoris* X-33 using a heat shock method at 42 °C for 2 min and the cells were screened on YPD agar plates containing 40 μ g mL⁻¹ zeocin. Transformants carrying the AP coding sequence was identified by PCR amplification using the previous primers and verified by nucleotide sequencing (Yegin and Fernandez-Lahore, 2013).

2.5. Cultivation

Colonies from *P. Pastoris* X-33 strain (control) and *P. Pastoris* X-33 aspartic protease (X-33 AP) were cultivated on YPD broth at pH 5 and pH 7 and incubated overnight at 30 °C. For protein expression, 1 mL of X-33-AP was added into 3 flasks containing 75 mL of YPD (pH 5) media and 1 mL of X-33 was added in 1 flask with 75 mL of YPD (pH 5) media. The same was done for YPD media with pH 7. The flasks were incubated at 30 °C for 6 days in a shaker incubator at 225 rpm. Samples were collected on 2nd, 4th and 6th days and centrifuged at 4000 rpm and 4 °C for 30 min. Then, the supernatant was used as a crude enzyme (Yegin and Fernandez-Lahore, 2013).

2.6. Milk clotting activity

The milk-clotting activity of the enzyme was undertaken according to (Arima *et al.*, 1970). Accordingly, 0.1 mL of the crude enzyme was added to 1 mL of reconstituted skim milk (Nestle TM) in 10 mL test tubes pre-incubated at 35 °C for 10 min. Reconstituted skim milk (NestleTM) solution consisted of 10 g dry skim milk/100 mL, 0.01 M CaCl₂ (AppliChemTM). The appearance of the first clotting flakes was visually evaluated and quantified in terms of Soxhlet units (SU). The endpoint was recorded when discrete particles were discernible. The clotting time T (s), the period of time starting from the addition of crude enzyme to the appearance of the first clots and the clotting activity was calculated using the following formula:

$$SU = (2400 * 5 * D) / (T * 0.5)$$

Where T = clotting time (s) D= dilution of crude enzyme

One SU is expressed as the quantity of enzyme required to clot 1 ml of a solution comprising 0.1 g skim milk powder and 0.01M calcium chlorides at 35 °C within 40 min.

2.7. Protein determination

Protein was determined according to the Bradford procedure utilizing bovine serum albumin as the standard (Yegin and Fernandez-Lahore, 2013).

2.8. SDS-PAGE Analysis

When necessary, the crude enzyme extracted from recombinant *P. pastoris* was concentrated at room temperature using a Vacuum Concentrator 5305 (Eppendorf, Hamburg, Germany). Then, 40 µL of the samples (concentrated and non-concentrated) were loaded on a 12.5% SDS–polyacrylamide gel. For the molecular marker, 10 µL of Colour Protein Standard (New England Biolabs TM) was loaded. Electrophoresis was run for 50 min at 300 V and 60 mA. The gel was then stained with Coomassie Brilliant Blue (AppliChem TM) overnight and then destained overnight with the destaining solution (Kangwa *et al.*, 2018).

2.9. Data analysis

Data analyses were performed using SAS software version 9 (Inc. Cary NC USA). The experiments were carried out in duplicate. Mean comparisons were done by Duncan's multiple range tests at the p-value of 0.05.

3. Results And Discussion

3.1. cDNA synthesis and amplification of aspartic protease gene

The concentration of RNA and aspartic protease gene was 140.1 ng/µL and 75.4 ng/µL, respectively. The amplified aspartic protease gene (fragment) had a size of 1.2 kbp (Fig.3.1). Similarly, the gene encoding milk-clotting aspartic protease (MCAP) from *M. circinelloides* strain DSM 2183 (Antonio *et al.*, 2013), MCAP protein from *M. circinelloides* DSM 2183 (Kangwa *et al.* 2018), yak chymosin from Yak (Luo *et al.* 2016) and aspartic proteinase from *M. mucedo* DSM 809 (Yegin and Fernandez-Lahore, 2013) were 959 bp, 1229 bp, 1,008 bp and 1,200 bp long, respectively.

The purified gene of interest was later sent for sequencing to Eurofins Genomics with the primers used in the gene amplification. The sequence results were analyzed in comparison to the gene sequence of aspartic protease from *Aspergillus oryzae* RIB40. Accordingly, the amino acid sequence alignment of the aspartic protease gene from *Aspergillus oryzae* DRDFS 13 showed 98% similarity with the aspartic protease gene from *A. oryzae* RIB40 (Fig. 3.2).

This confirmed the presence of catalytic Asp residues in the amplified gene from *A. oryzae* DRDFS13 (Antonio *et al.*, 2013). Similarly, the deduced amino acid sequence for milk-clotting acid protease (MCAP) from *M. circinelloides* showed 88% similarity with *M. bacilliformis* PEP A gene (Antonio *et al.*, 2013). The deduced amino acid sequence of the PEPA gene from *A. oryzae* has also shown 67% homology to the PEP A gene of *A. awamori* (Gomi *et al.*, 1993). In another study, the gene encoding aspartic proteinase of *M. mucedo* had 80 % similarity with the *Rhizopus niveus* gene for aspartic proteinase II and 73 % similarity with rhizopuspepsinogen precursor of *Rhizopus microsporus* var. *chinensis* (Yegin and Fernandez-Lahore, 2013).

3.2. Cloning and expression of aspartic proteinase gene in *pastoris*

The concentrations of pGAPZαA vector (Fig.3.3 and expression vector pMK-AP (Fig.3.4) were determined by NanoDrop 2000 and found 186.4 ng/ μL 55.8 ng/ μL.

The aspartic proteinase gene from *Aspergillus oryzae* DRDFS 13 was successfully expressed in *P. pastoris* under the control of the GAP promoter. Antonio *et al.*, (2013), reported the expression of the aspartic protein gene from *M. circinelloides* in *P. pastoris* under the control of the constitutive GAP promoter. Similarly, the gene encoding an aspartic protease (MCAP) from *M. circinelloides* DSM 2183 cloned in *P. pastoris* was successfully expressed using both the native *M. circinelloides* signal peptide (mcSP) and α-factor secretion signal from *Saccharomyces cerevisiae* (α-MF) (Kangwa et al. 2018).

A novel aspartic protease gene (RmproA) from *Rhizomucor miehei* CAU432 cloned into *P. pastoris* was also successfully expressed in *P. pastoris* (Sun et al. 2018). In another study, an alkaline protease gene from *A. oryzae* and a serine protease gene from *Thermoascus aurantiacus* var. *levisporus* were cloned in *P. pastoris* GS115 were fruitfully expressed in *P. pastoris* (Guo & Ma, 2008; Li *et al.*, 2011).

3.3. Milk clotting activity

The milk clotting activity of the crude extract collected from *P. pastoris* X-33 AP (transformed) and *P. pastoris* X-33 (wild type, control) was determined at day 2, 4 and 6 days. The highest MCA (190.47 U/mL) and specific activity (30.75 U/mg) of the secreted crude enzyme from the recombinant yeast were obtained at pH 5 after 6 days of incubation (3.1). Similarly, the maximum MCA recorded for recombinant enzyme (210 U/mL) from *M. mucedo* DSM 809 expressed in *P. pastoris* (Yegin and Fernandez-Lahore, 2013) and the recombinant MCAP (257 CU/mL) from *M. circinelloides* expressed in *P. pastoris* (Antonio et al. 2013) was comparable with this study. However, the milk-clotting activity recorded from recombinant *P. pastoris* with plant milk-clotting aspartic protease (23 CU) (Feijoo-siota et al. 2018) and recombinant *P. pastoris* with bovine chymosin B (96 IMCU/mL) (Nosedá et al. 2013) was lower than the present study. In another study, high protease activity (3480.4 U/mL) was also recorded from an extracellular protease extracted from recombinant *P. pastoris* (Sun et al. 2018).

The data also showed substantial milk-clotting activity (MCA) of the recombinant yeast was obtained at pH 5; whereas, the crude enzyme from recombinant yeast cultivated on YPD media at pH 7 did not possess milk-clotting activity. This could be due to the fact that aspartic proteases may be produced under acidic conditions indicating that the initial pH of the media plays an important role in protein expression. Antonio *et al.*, (2013) also reported the highest milk-clotting activity for aspartic protease from recombinant yeast (X-33/pGAPZα+MCAP-5) in YPD medium at pH 5.0. Interestingly, the enzyme was even induced in *Pichia pastoris* X-33 from *M. mucedo* DSM 809 at an initial medium pH of 3.5 (Yegin and Fernandez-Lahore, 2013). On the other hand, maximum enzyme activity was detected from recombinant *P. pastoris* at 72 h showing a difference in activity as a function of time (Antonio *et al.*, 2013; Yegin and Fernandez-Lahore, 2013). The differences in the time of maximum milk-clotting activity production could be due to the media and physicochemical parameters used for cultivation of the recombinant yeast.

The milk-clotting activity and specific activity of the transformed crude enzyme increased as the fermentation time increased. However, the total protein content of the crude enzyme did not show significant differences at the time of incubation increased. However, there is a slight increase in protein concentration for the crude enzyme extracted from the control on the 6th day (Table 3.1, Fig. 3.5). Comparatively, a slightly higher protein concentration from recombinant yeast was noticed at 96 h of fermentation time. Similarly, the cultivation of recombinant *P. pastoris* under optimized conditions produced a maximum protein concentration at 72 h. Likewise, the cultivation of recombinant *P. pastoris* under optimized conditions produced maximum protein concentration at 72 h (Yegin and Fernandez-Lahore, 2013).

Table 3.1: The TP, MCA and specific activity of the crude enzyme from *P. pastoris* X-33 AP (transformed) at different pH

Microbes	Initial Media pH	Cultivation time (days)	MCA (U/mL) Mean±STD	Total protein (mg/mL)	Specific Activity (U/mg)
Recombinant <i>P. pastoris</i> X.33 AP	5.00	2	43.63±0.03 ^e	6.10	7.14
	7.00	2	ND ^f	5.84	-
	5.00	4	92.31±0.30 ^b	6.24	14.79
	7.00	4	ND ^f	5.51	–
	5.00	6	190.47±0.20 ^a	6.19	30.75
	7.00	6	ND ^f	5.44	-

*ND- milk clotting activity not determined within 40 min, STD: standard deviation, Mean: is average of two measurements, Different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05).

3.4. SDS-PAGE analysis

The SDS-PAGE analysis showed that the major protein expressed by recombinant yeast *P. pastoris* X-33 AP has a molecular mass between 32 and 46 kDa (Fig. 3.6B). However, some other proteins with molecular mass above 80 kDa were also observed on the SDS-PAGE. This may infer to be either from the proteins contained in the media, or some other proteins expressed apart from the protein of interest. Similarly, the SDS-PAGE analysis for the recombinant Rmap (extracellular aspartic proteinase) secreted by *P. pastoris* transformants, MCAP secreted by recombinant yeast X-33/pGAPZα⁺- SyMCAP-6, MpAPr1 (an aspartic protease gene) secreted by recombinant *Komagataella pastoris* X33, *Mucor pusillus* rennin expressed in *P. pastoris*, recombinant chymosin in *P. pastoris* and progaline B (novel plant milk-clotting aspartic protease) in *P. pastoris* showed a single band of approximately 33.5 kDa (Schoen *et al.*, 2002),

between 33 kDa and 37 kDa (Antonio et al. 2013), 43.3 kDa (Theron and Divol 2017), 44 KDa (Beldarraín et al. 2000), between 30 to 45 kDa (Nosedá et al., 2013) and 48 kDa (Feijoo-siota *et al.*, 2018), respectively.

However, the recombinant aspartic proteinase expressed in *P. pastoris* with a molecular mass of 52.4 kDa (Sun et al. 2018) and 46–58 kDa (Yegin and Fernandez-Lahore, 2013) by SDS-PAGE was different from the present study.

Conclusions

Based on the results it can be concluded that the aspartic protease gene from *Aspergillus oryzae* DRDFS 13 cloned and expressed in *P. pastoris* X-33 AP was a functionally active protein with significant milk-clotting activity. Therefore, the milk-clotting protease extracted from the recombinant yeast may be a suitable candidate for application in cheese and other food industries.

Declarations

Ethics approval and consent to participate

Non-applicable

Consent for publication

Non-applicable

Availability of data and materials

All data generated or analyzed during this study are publicly available and included in this published article.

Competing interests

The authors declare that they have no competing interests.

Fund

This work was supported by Microbial, Cellular and Molecular Biology, Addis Ababa University.

Authors'contributions

JM: Contributes to conception, acquisition and analysis, interpretation of data, and drafted the work. MK: Contributes to conception, acquisition and analysis, interpretation of data, and drafted the work. KK: Contributes to conception, acquisition and analysis, interpretation of data, and drafted the work. FA: analysis and substantively revised the work. HMFL: contributes to conception, acquisition and analysis, interpretation of data, and drafted the work. All authors have approved the submitted version and the

modified version that involves the author's contribution to the study; and have agreed both to be personally accountable for the author's own contributions and ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved and the resolution documented in the literature.

Acknowledgment

Moreover, we would like to acknowledge the Department of Microbial, Cellular and Molecular Biology of Addis Ababa University and Downstream Processing Laboratory, Department of Life Sciences and Chemistry, Jacobs University, Bremen, Germany for the facilitation of laboratory space and provision of basic facilities.

References

- Ahmad, Mudassar, Melanie Hirz, Harald Pichler, and Helmut Schwab. 2014. "Protein Expression in *Pichia Pastoris*: Recent Achievements and Perspectives for Heterologous Protein Production." *Applied Microbiology and Biotechnology* 98: 5301–17. doi:10.1007/s00253-014-5732-5.
- Antonio, Jose, Gama Salgado, Martin Kangwa, and Marcelo Fernandez-lahore. 2013. "Cloning and Expression of an Active Aspartic Proteinase from *Mucor Circinelloides* in *Pichia Pastoris*." *BMC Microbiology* 13 (1). BMC Microbiology: 1. doi:10.1186/1471-2180-13-250.
- Arima, k., J. Yu, and S. Iwasaki. 1970. "Milk-Clotting Enzyme from *Mucor Pusillus* Var. Lindt." *Methods in Enzymology* 19: 446–59.
- Beldarraín, Alejandro, Niuris Acosta, Raquel Montesinos, Marlene Mata, and José Cremata. 2000. "Characterization of *Mucor Pusillus* Rennin Expressed in *Pichia Pastoris*: Enzymic, Spectroscopic and Calorimetric Studies." *Biotechnology and Applied Biochemistry* 31 (2): 77–84. doi:10.1042/ba19990094.
- Cereghino, Joan Lin, and James M Cregg. 2000. "Heterologous Protein Expression in the Methylophilic Yeast *Pichia Pastoris*." *FEMS Microbiology Reviews* 24: 45–66.
- Cregg, James M, Joan Lin Cereghino, Jianying Shi, and David R Higgins. 2000. "Recombinant Protein Expression in *Pichia Pastoris*." *Molecular Biotechnology* 16: 23–24.
- Feijoo-siota, Lucía, José Luis, R Rama, Angeles Sánchez-pérez, and Tomás G Villa. 2018. "Expression , Activation and Processing of a Novel Plant Milk-Clotting Aspartic Protease in *Pichia Pastoris*." *Journal of Biotechnology* 268 (October 2017). Elsevier: 28–39. doi:10.1016/j.jbiotec.2018.01.006.
- Gomi, Katsuya, Kenji Arikawa, Naokata Kamiya, and Katsuhiko Kitamoto. 1993. "Cloning and Nucleotide Sequence of the Acid Protease-Encoding Gene (pepA) from *Aspergillus Oryzae*." *Biosci. Biotech. Biochem.*, 57 (7): 1095–1100. doi:10.1271/bbb.57.1095.

- Guo, Ji-ping, and Ying Ma. 2008. "High-Level Expression, Purification and Characterization of Recombinant *Aspergillus Oryzae* Alkaline Protease in *Pichia Pastoris*" 58: 301–8. doi:10.1016/j.pep.2007.12.005.
- Kangwa, Martin, Jose Antonio, Gama Salgado, Hector Marcelo, and Fernandez Lahore. 2018. "Identification and Characterization of N - Glycosylation Site on a *Mucor Circinelloides* Aspartic Protease Expressed in *Pichia Pastoris*: Effect on Secretion, Activity, and Thermo - Stability." *AMB Express* 8 (157). Springer Berlin Heidelberg: 1–13. doi:10.1186/s13568-018-0691-3.
- Li, An-Na; Xie, Chen; Zhang, Jia and Li, Duo-Chuan. 2011. "Cloning, Expression, and Characterization of Serine Protease from Thermophilic Fungus *Thermoascus Autranthiacus* Var. *Levisporus*." *The Journal of Microbiology* 49 (1): 121–22. doi:10.1007/s12275-011-9355-6.
- Luo, Fan, Wei Hua Jiang, Yuan Xiao Yang, Jiang Li, and Ming Feng Jiang. 2016. "Cloning and Expression of Yak Active Chymosin in *Pichia Pastoris*." *Asian Australas. J. Anim. Sci.* 29 (9): 1363–70.
- Nair, I. C., and K. Jayachandran. 2019. "Aspartic Proteases in Food Industry." In *Green Bio-Processes, Enzymes in Industrial Food Processing*, edited by Varjani; Sindhu Raveendran Parameswaran, Binod; Sunita, 15–30. Singapore: Springer Nature.
- Nosedá, Diego Gabriel, Matías Nicolás Recúpero, Martín Blasco, Gastón Ezequiel Ortiz, and Miguel Angel Galvagno. 2013. "Cloning, Expression and Optimized Production in a Bioreactor of Bovine Chymosin B in *Pichia* (*Komagataella*) *Pastoris* under AOX1 Promoter." *Protein Expression and Purification* 92 (2). Elsevier Inc.: 235–44. doi:10.1016/j.pep.2013.08.018.
- Schoen, C, U Reichard, M Monod, H D Kratzin, and R Ru. 2002. "Molecular Cloning of an Extracellular Aspartic Proteinase from *Rhizopus microsporus* and Evidence for Its Expression during Infection." *Medical Mycology* 40 (June 2001): 61–71.
- Sun, Qian, Fusheng Chen, Fang Geng, Yongkang Luo, Siyi Gong, and Zhengqiang Jiang. 2018. "A Novel Aspartic Protease from *Rhizomucor Miehei* Expressed in *Pichia Pastoris* and Its Application on Meat Tenderization and Preparation of Turtle Peptides." *Food Chemistry* 245 (October 2017). Elsevier: 570–77. doi:10.1016/j.foodchem.2017.10.113.
- Theron, Louwrens Wiid, and Benoit Divol. 2017. "Characterisation of the Enzymatic Properties of MpAPr1, an Aspartic Protease Secreted by the Wine Yeast *Metschnikowia Pulcherrima*." *J Sci Food Agric* 97 (August 2016): 3584–3593. doi:10.1002/jsfa.8217.
- Yegin Sirma; and Fernandez-Lahore, M. 2013. "A Thermolabile Aspartic Proteinase from *Mucor Mucedo* DSM 809: Gene Identification, Cloning, and Functional Expression in *Pichia Pastoris*." *Mol Biotechnol* 54: 661–72. doi:10.1007/s12033-012-9608-6.

Figures

Figure 1

Agarose gel electrophoresis of marker protein and PCR product for aspartic protease gene. bands on molecular marker indicated kilobases, Lane M is the marker protein, Lane 1 and 2: Negative control Lane 3: aspartic protease gene

Figure 2

Amino acid sequence alignment of the aspartic protease gene. DRDFS AP: the amino acid sequence of aspartic protease gene from *Aspergillus oryzae* DRDFS13. *A. Oryzae* RIB40: the amino acid sequence of aspartic protease gene from *A. Oryzae* RIB40.

Figure 3

Representation of pGAPZaA vector including the site of the restriction enzymes XhoI and NotI used for cloning.

Figure 4

Schematic representation of the pMK-AP vector used for protein expression.

Figure 5

The protein concentration of the crude enzyme from transformant P. Pastoris X-33 AP and the control P. Pastoris X-33 at pH 5 and 7

Figure 6

Depicts the SDS-PAGE gel of crude enzyme protein extracted from the recombinant *P. pastoris* and concentrated by vacuum concentrator after Coomassie Blue Brilliant staining. The molecular marker is shown in lane 1, and the lanes 2-9 are loaded with concentrated samples from day 2 pH5, pH7, day 4 pH5, pH7, day 6 pH5, pH7 and day 6 controls pH5 and pH. The black arrows in lanes 2, 4 and 6 indicate the bands between 32 and 46 kDa

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.docx](#)