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Research Article

# Characterization of the Crude Alkaline Extracellular Protease of *Yarrowia lipolytica YlTun15*

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**Abstract:** *Yarrowia lipolytica YlTun15* (GeneBank Acc. N° MF327143), *isolated* from farmed *Dicentrarchus labrax*'s gills, secretes an alkaline extracellular protease, which exhibited the highest activity at pH 9 and temperature 45°C. The enzyme activity extracted was tested in the presence of different ion metal and protein inhibitors. The enzyme activity increased in the presence of both $Cu^{2+}$ (1 mM) and $Mn^{2+}$ (5mM) in the medium. $K^{+}$, $Na^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ had no effect on the enzyme activity while $Ni^{+}$, $Hg^{+}$, $Zn^{2+}$ and $Fe^{2+}$ decreased significantly its relative activity to 43.63%, 66.25%, 30.75% and 19.48% respectively at the 5 mM level. The enzyme was almost (activity=1.47%) inhibited by phenylmethylsulfonyl fluoride at the concentration of 5 mM. However, the protease activity was relatively constant in the presence of EDTA and SDS that may conclude that this enzyme was not a metalloprotease and belong to the serine protease category. After 18 months-storage at -20°C, the enzyme activity has decreased to 23.17%. This protease may have a potential application in food and detergent activity.

**Keywords:** *Yarrowia lipolytica*; Alkaline extracellular protease; Marine yeast; Enzyme

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# Introduction

Along the vast biodiversity of animals, plant and seaweeds; marine environments contain innumerable microorganisms including microalgae and yeast each; with distinctive metabolic functions capable of producing bio-actives substances. The marine yeasts; initially discovered and isolated from the Atlantic Ocean (van Uden and Castelo-Branco, 1963); were subsequently isolated from various sources including seawater, seaweeds, fish, and marine sediments (Kutty and Philip, 2008). Beside its nutritional quality and its possible utilization as animal or aquaculture feed, marine yeast represent a substantial and a competitive source of enzymes. The global market for industrial enzymes is now well established reaching around $5.0 billion in 2016 with a potential for growth rate of 4.7% until 2021 (Dewan, 2017). With several distinctive and promising attributes (Zaky et al., 2014), marine yeasts produce various enzyme such as inulinase, amylase, cellulase, exo β-1,3-glucanase, and protease (Liu et al., 2008; Zhang et al., 2009; Ni et al., 2009; Peng et al., 2011; Xu et al., 2012; Zhang et al., 2015; Rong et al., 2015 and Chi et al., 2016).

Proteases are considered among the most important groups of industrially and commercially produced enzyme with application in various sectors such as biotechnology, food, pharmaceutical process, cosmetics, and detergent (Anwar et al., 1998; Bagewadi *et al*., 2011; Vermelho *et al*., 2012; Alnahdi., 2012; Kasana et al., 2011; Pokora et al., 2017).

*Yarrowia lipolytica* is one of the most extensively studied ‘‘non-conventional’’ yeasts which is currently used as a model for the study of protein secretion, peroxisome biogenesis, dimorphism, degradation of hydrophobic substrates, and several emerging fields (Fickers et al., 2005). According to Singhal (2012) and Ogrydziak (2013), all *Yarrowia lipolytica* strains can produce acid and alkaline extracellular protease with a high rate of alkaline extracellular (1-2% of total cell protein) (Motabe et al., 1988; Madzak et al., 2004). In a previous study (Bessadok et al., 2015), proteases secreted by the strain of *Yarrowia lipolytica* YlTun15, was extracted and its alkaline nature was determined. The aim of the current study is to characterize the alkaline extracellular protease.

# Materials and Methods

## Determination of the protease activity

Yeast *Yarrowia lipolytica* YlTun15 cell culture was centrifuged at 8000 rpm/4°C/30 min and the resultant supernatants were concentrated (Amicon Bioseparation, Merck). Thereby, 0.5 ml of the supernatant was mixed with 1 ml of 0.5% of casein solution in glycine-NaOH buffer (0.05M, pH 9.0). The mixture was incubated at 45°C for 30 min and 2 ml of trichloroacetic acid (10%) was added to the mixture immediately to stop the reaction. The reaction mixture was centrifuged at 10000 rpm/4°C (Ma et al., 2007 and Chi et al., 2007). Tyrosine content was determined calorimetrically at 650 nm according to Lowry method (1951). The enzyme activity was defined as the amount of the enzyme that liberated 1 µg of tyrosine per minute. Protein concentration was measured by the method of Bradford (Bradford, 1976).

## Effect of pH and temperature on enzyme activity and the stability

The effect of pH on the purified alkaline protease activity was determined by incubating the purified enzyme at pH 6 to 10 using the standard assay conditions. The buffers used were 0.1 M sodium phosphate (pH 6.0 to 8.0), 0.1M glycine-NaOH (pH 9 to 10). The optimal temperature for the enzyme activity was determined at 15°C, 25°C, 45°C and 60°C in the same buffer as described in the first section. The pH stability was tested via 12 h pre-incubation of the crude enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 6 to 10 at 4°C. The activities of protease were measured immediately after this treatment with the standard method as mentioned previously. Temperature stability of the purified enzyme was tested by pre-incubating the enzyme at different temperatures (15°C, 25°C, 45°C and 60°C) for 1h, the relative activity was immediately measured (Ma et al., 2007).

## SDS-PAGE electrophoresis

The profile of the protease was analysed in non-continuous denaturing Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis SDS-PAGE (Laemmli, 1970) in a mini Protean II Electrophoresis System (Bio-Rad, Hercules, CA.). 5 µl of protein molecular weight marker of 250 KDa (Precision Plus Protein ™ Dual Xtra standard; Criterion 4-20%Tris-HCl; Bio-Rad) was added into gel. The gel was than stained using Coomasie Brilliant Blue (R-250) method ans analysed with Digi-Doc-IT System (UVP, Upland, Ca).

## Effect of different metal ions on the enzyme activity

To examine the effects of the different metal ions on the protease activity, enzyme assay was made for 1 h in the reaction mixture as described previously with various metal ions at a final concentration of 1 mM to 5 mM. The activity assayed in the absence of metal ions was defined as the control. The metal ions tested included $Zn^{2+}$, $Cu^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Na^{2+}$ $K^{+}$, $Hg^{+}$ and $Ni^{+}$ (Ma et al., 2007).

## Effect of protein inhibitors on the enzyme activity

The effects of inhibitors on protease activity were measured in the reaction mixture. Such inhibitors included ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and sodium dodecylsulfate (SDS) at a final concentration ranging from 1 to 5 mM, respectively. The crude enzyme was pre-incubated with the respective compound for 30 min at 0°C, followed by the standard enzyme assay as described in the preceding text. The activity assayed in the absence of the protein inhibitors was defined as the control (Chi et al., 2007).

### Effect of storage time on the enzyme stability

The storage stability of the enzyme at -20°C was analyzed as described by Beena et al. (2012). The enzyme was stored at the corresponding temperature and the residual activity was recorded by the standard assay procedure as described previously after exactly 4 months for a period of 18 months. The relative activity at day one was considered as 100%.

## Statistical analysis

Data were subjected to analyses of variance at the 5% level using SPSS 24.0 software and the *Tuckey*-test was performed to separate differences among means.

## Results and Discussions

### Gel electrophoresis

The profile of the protein was determinate with SDS-PAGE. **Figure 1** shows that the purified enzyme presented two bands but after a dilution (1:4), only one band persisted with the same intensity. Thus, the crude alkaline extracellular enzyme has a relative molecular weight about 35 kDa. According to Matoba and Ogrydziak (1989); Barth and Gaillardin (1997), *Yarrowia lipolytica* can produce an Alkaline extracellular protease with a molecular weight of 32 kDa which processed from a 55 kDa glycosylated precursor. It was also mentioned that the molecular weight of alkaline protease secreted by microorganism ranged between 15 to 45 kDa (Kumar and Takagi, 1999).

### Effect of temperature on the stability and the optimum activity of the enzyme

In this study, the *Yarrowia lipolytica* alkaline protease activity measured at different temperatures ranging from 15°C to 60°C **(Figure 2)**; revealed a maximum activity at 45°C. Similar result

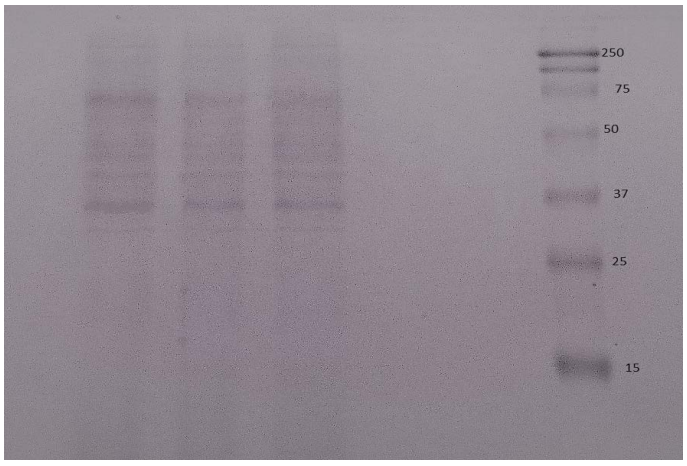


**Figure 1:** SDS-PAGE of the crude protease. The lanes are as follows: L1: marker protein with a molecular weight indicated in right (MW: kDa); L2: purified protease; L3 and L4: purified protease diluted 4X in buffer sample (60 mM Tris-HCl (pH: 6.8); 25% glycerol; 2% SDS; 0.1% Bromophenol Bleu; 14.4 mM β-Mercaptoethanol).

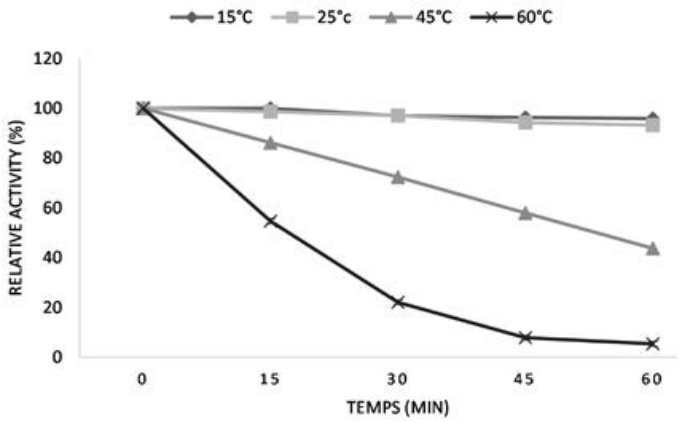


**Figure 2:** *Yarrowia lipolytica* crude protease: Effect of different temperatures (15°C, 25°C, 45°C and 60°C) on the extracellular alkaline protease stability. (Data are given as means ± SD; (n=3 for each assay).

was reported for *Aureobasidium pullulans* (Chi et al., 2007; Ma et al., 2007), but a lower temperature (37°C) for optimal protease activity was found *B. subtillus* (Alam et al. 2017). The residual protease activities were 97.29 ± 0.11% and 95.85 ± 0.34% at 15 and 25°C respectively. From the temperature of 45°C, the relative activity drops significantly and the enzyme becomes almost inactive at 60°C in 30 minutes. Such result suggests that this enzyme is thermosensitive at temperature above 45°C but is stable at temperature ranging from 15-25°C.

### Effect of pH on the stability and the optimum activity of the enzyme

It well established that pH is a key factor influencing the ionization state of the amino acid or ionization of the substrate and thereby the expression of the enzyme activity (Bezawada et al., 2011). In the present study, the relative protease activity measured at different pH ranging from 6 to 10 **(Figure 3)** showed maximum activities at pH 9 (88.69 ± 1.71). In this case, the enzyme stability was maintained over 12 h at 4°C. The average residual activity of the extracted protease showed a linear and significant decrease with pH drop. This result is consistent with that found for protease extracted from other micro-organisms (Kumar and Tagaki, 1999; Ma et al., 2007), but a higher pH value (11) was found for an optimal activity of protease from *Aspergillus terreus* (Niyonzima and More, 2015), which was also inactive at low pH. It was also reported that a large amount of alkaline protease could be secreted under neutral condition and three acids protease can be secreted by *Y. lipolytica* (Barth and Gaillardin, 1997). Moreover, Li et al. (2010) report that despite *M. reukaufii* W6b was isolated from alkaline marine environment (pH 8), it is completely unknown if this strain can produce this enzyme in natural sea sediment.

### Effect of metal ion on the enzyme activity

The result presented in **Table 1** shows that $K^+$, $Na^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ had no effect on the protease activity. Some of the metal ions such as $Ca^{2+}$, $Mn^{2+}$, and $Mg^{2+}$ increased and stabilized

protease activity; this may be due to the activation by the metal ions (Bezawada et al., 2011; Kumar et Takagi, 1999). However, **Figure 4** illustrate that metal ions including $Ni^{+}$, $Zn^{2+}$ and $Fe^{2+}$ showed maximum inhibition on enzyme activity up to 60%, while $Hg^{+}$ inhibited the enzyme activity up to 35%. Analogous results were found for the *A. pullulan*, *A. terreus* and *B. subtilis* (Ma et al., 2007, Niyonzima and More, 2015, and Alam et al., 2017).The inhibitory effect of mercury was due to the fact that it reacts with protein thiol groups and with histidine and tryptophan residues (Bezawada et al., 2011, Barth and Gaillardin, 1997). The $Cu^{2+}$ and $Mn^{2+}$ cations seem to have an antagonistic effect on the enzyme activity **(Figure 5)**.

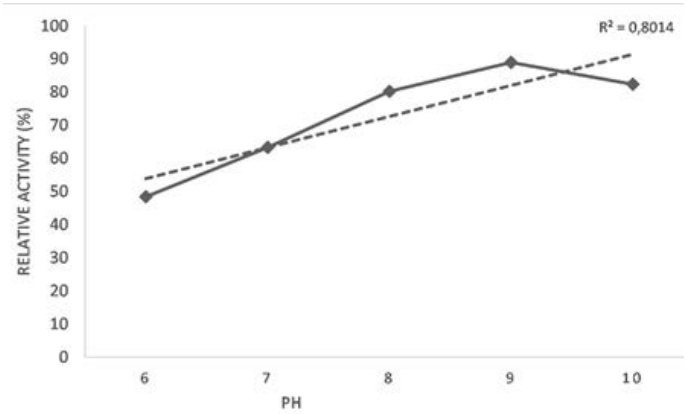


**Figure 3:** *Yarrowia lipolytica* protease: Effect of different pH on the stability of the extracellular alkaline protease (6, 7, 8, 9 and 10) after 12 h of incubation in appropriate buffer. (Data are given as means ± SD; n=3 for each assay, result are treated with SPSS).

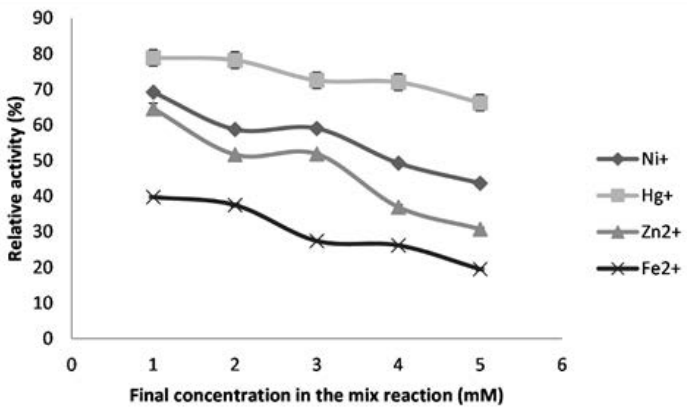


**Figure 4:** *Yarrowia lipolytica* crude protease: Effect of Cations on the extracellular alkaline protease activity of marine yeast *Y. lipolytica* (Data are given as means ± SD; n=4 for each assay, results treated with SPSS).

## Effect of the protein inhibitors

Yeast alkaline protease activity reacted differently with the three tested inhibitors **(Figure 6)** with PMSF having the highest inhibitory effect at the lowest concentration of 2 mM in the broth. With a concentration of 5 mM PMSF in the broth, a drastic inhibition of the protease activity occurred (1.47%). Such results confirm that this extracellular enzyme was a serine protease (Castagliuolo et al., 1996; Moteba et al., 1997; Barth and Gaillardin, 1997; Alam et al., 2017) and it is belong to subtilism family. Up to 5 mM, the protease activities in the presence of EDTA and SDS were 74% and 55% respectively. Correspondingly, proteases from

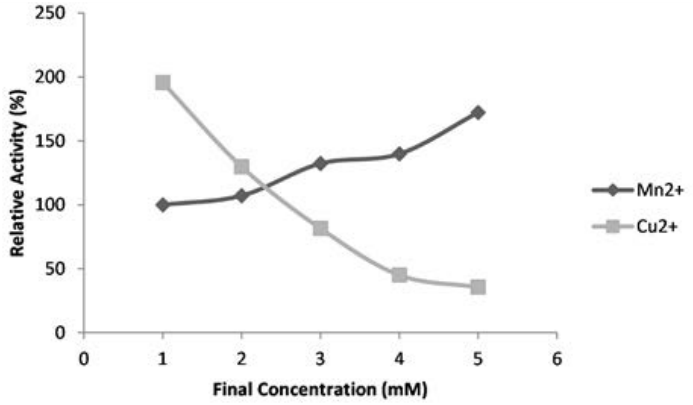


**Figure 5:** *Yarrowia lipolytica* crude protease: Effect of $Cu^{2+}$ and $Mn^{2+}$ on the extracellular alkaline protease activity of marine yeast *Y. lipolytica* (Data are given as means ± SD; n=4 for each assay, results treated with SPSS).

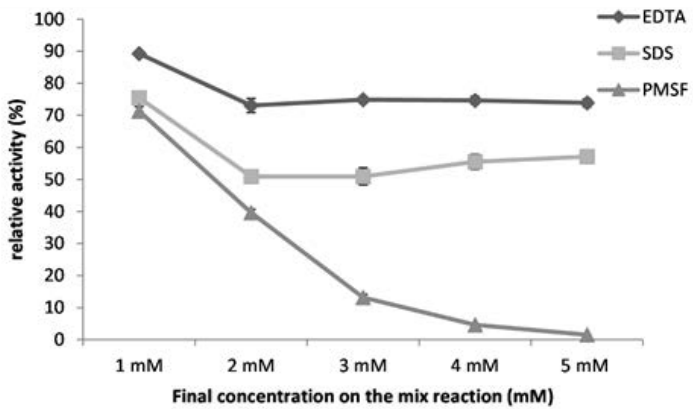


**Figure 6:** *Yarrowia lipolytica* crude protease: Effect of protein inhibitors on the extracellular alkaline protease activity of marine yeast *Y. lipolytica* (Data are given as means ± SD; n=4 for each assay, results treated with SPSS).

**Table1:** *Yarrowia lipolytica* protease: Effect of Cations on the relative activity of the crude extracellular.

| | Control | 1 mM | 2 mM | 3 mM | 4 mM | 5 mM |
|---|---|---|---|---|---|---|
| **$K^{+}$** | 100.86 ± 1.92 | 101.08 ± 0.97 | 101.13 ± 0.99 | 98.94 ± 1.37 | 99.42 ± 0.69 | 98.8 ± 2.31 |
| **$Na^{2+}$** | 100.86 ± 1.92 | 98.66 ± 1.66 | 101.23 ± 1.21 | 99.40 ± 0,82 | 98.8 ± 1.97 | 100 ± 0.11 |
| **$Mg^{2+}$** | 100.86 ± 1.92 | 101.26 ± 1.32 | 107.66 ± 1,36 | 98.66 ± 1.68 | 103.9 ± 1.97 | 109.85 ± 1.35 |
| **$Ca^{2+}$** | 100.86 ± 1.92 | 101.75 ± 2.04 | 100.61 ± 0.09 | 99.52 ± 2.39 | 100.61 ± 1.93 | 108.04 ± 3 |

*Aspergillus terreus* and *Aspergillus tamari* exhibited a stability in the presence of EDTA (Anandan et al., 2007, Niyonzima and More, 2015). Such finding approve that *Yarrowia lipolytica* protease do not belong to the metalloprotein group, and could be of interest to the detergent industry as the stability of protease in the presence of chelating agents such as EDTA is a prerequisite for any detergent enzyme (Beg and Gupta, 2003).

### Effect of storage on the stability of the enzyme activity

Monitoring the effect of storage at -20°C for one year on protease activity showed a significant decrease in relative activity to reach 23.17 ± 2.11 in January 2017. This decrease might be explained by the ice crystal formation that inactivates enzymes (Beena et al., 2012). Comparing to this result, Niyonzima and More (2015) found that for *Aspergillus terreus* the protease decrease with 9% after 40 days storage in -20°C.

## Conclusion

The purified extracellular alkaline protease extracted from *Yarrowia lipolytica* YlTun15 shows promising proprieties of stability and activities that may be used in profile various applications. Further, this study will be completed with proteomic analysis to sequence and well characterize this enzyme.

## Acknowledgment

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