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Heterologous Expression of *Bacillus pumilus* 3–19 Protease in *Pichia pastoris* and Its Potential Use as a Feed Additive in Poultry Farming

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Abstract
Proteases are one of the most innovative products used to improve the efficiency of feed additives. Due to their hydrolytic properties, they enhance the absorption of amino acids, which can reduce the protein content and the cost of feeds. The subtilisin-like proteinase of *Bacillus pumilus* 3–19 is a promising candidate for industrial use as a feed additive. However, in order to obtain a high yield of the enzyme, it is necessary to develop a highly efficient expression system. The aim of the study was to obtain stable expression of the optimized *B. pumilus* 3–19 protease gene in the *Pichia pastoris* expression system and evaluate the correlation of enzyme activity with the choice of vector type and signal peptides. The efficient secretion of subtilisin-like protease into the culture fluid of the recombinant yeast strains was confirmed. The study showed that the incubation time affects the synthesis of protease in *P. pastoris*, and the maximum activity of the enzyme was observed at 72 h of growth of the yeast culture. Yeast strains with constructs based on the low-copy vector pPINK-LC showed higher protease activity (U/mL) in the hydrolysis of azocasein (2.63 ± 0.16 for killer signal peptide (SP), 2.49 ± 0.08 for α-mating factor presequence, 2.19 ± 0.11 for lysozyme SP) than strains with constructs based on the pPINK-HC vector (1.86 ± 0.09 for killer SP, 2.21 ± 0.07 for α-mating factor presequence, 1.31 ± 0.11 for lysozyme SP), regardless of which signal peptide was used. The ability of the recombinant protease to hydrolyze a specific substrate confirms that the enzyme is a member of the subtilisin family. The maximum protease activity was obtained for yeast strains with pPINK-LC-killer-*aprBp* (5.75 ± 0.08 U/mL) and pPINK-LC-α-mat.factor-*aprBp* (4.33 ± 0.07 U/mL) constructs. This study demonstrated that the subtilisin-like protease from recombinant *P. pastoris* strains exhibits proteolytic activity, which depends on the incubation time and the choice of signal peptide and vector. The production of bacillary protease by the heterologous yeast-based expression system makes this system promising for the development of new feed additives for animal husbandry.

Keywords Protease · *Bacillus pumilus* · Feed additive · Heterological expression system · *Pichia pastoris*

1 Introduction

The expected expansion of animal production, driven by the rising world population, is creating a global shortage of feed protein supply. Owing to this, feed protein has become one of the most expensive and limiting feed additives [1]. It is known that the biological fermentation of protein feeds, lowering of dietary protein using amino acids, and the application of proteases will be instrumental in solving this problem or at least decrease the demand and supply gap [2, 3].

Proteases are rated among the three largest groups of industrial enzymes and account for about 60% of the total global sale of enzymes, 40% of which belong to bacterial proteases [4]. Bacterial serine proteases have wide substrate specificity and high resistance to various conditions, which warrants their use in biotechnology, in particular as bio-additives for farm birds and animals [5]. Supplementing feeds with proteases enhances the absorption of amino acids by animals and saves funds for the purchase of synthetic amino acids [6, 7]. It has also been shown that the use of proteases as feed additives increases the bioavailability of nitrogen, which in turn reduces the levels of ammonia pollution in the soil [8]. Proteases facilitate the action of other enzymes and increase the cost-effectiveness of feeds, as well as promote the growth of beneficial microflora in the intestines of broilers [9]. They dissolve undigested proteins, thereby reducing the risk of

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