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Heterologous Expression of Histidine Acid Phytase from *Pantoea* sp. 3.5.1 in *Yarrowia lipolytica*

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Abstract
The dimorphic yeasts *Yarrowia lipolytica* are used as effective expression system and are characterized by a high level of production of heterologous protein. In this work, we aimed to clone and express *Pantoea* sp. 3.5.1 *agpP* phytase gene in *Yarrowia lipolytica*. Genetic constructs containing the native phytase gene (*agpP*) under the control of the strong hybrid promoter hp4d and the signal peptide of the alkaline extracellular protease XPR2 gene, as well as the optimized gene (*agpP-opt)* of phytase under the control of two signal peptides—bacterial and yeast were obtained. Recombinant *Y. lipolytica* strains with integrated bacterial phytase genes were obtained and expression of phytase was analyzed.

Keywords Phytase · Cloning · Heterologous expression · *Yarrowia*

1 Introduction

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) catalyze the stepwise hydrolysis of phosphate groups from phytic acid (*myo*-inositol hexakisphosphate) or its salt phytate. First phytases were reported in 1907 [1], and during the last 25 years, these enzymes have attracted the attention of scientists and biotechnologists because of their potential application in animal and human nutrition, agriculture, and medicine. Phytases catalyze the degradation of phytic acid into lower *myo*-inositol phosphates: pentakis-(IP5), tetrakis-(IP4), tris-(IP3), bis-(IP2), and monophosphate/s (IP1) with the release of inorganic orthophosphate (Pi) in sequential manner [2]. *myo*-Inositol and its phosphate derivatives are common in biology and have a multitude of functions across the various taxa, including roles in regulating ion-channel permeability, phosphate levels, metabolic flux, transcription, mRNA export and translation, insulin signaling, embryonic development, and the stress response [3]. Following the discovery of *myo*-inositol-1,4,5-trisphosphate as a second messenger, many other inositol phosphates were discovered in quick succession [4]. The production of *myo*-inositol phosphate intermediates has received much attention; however, chemical synthesis of these compounds is difficult. In contrast, an enzymatic synthesis has the advantage of high stereospecificity and mild reaction conditions [5]. It has been shown by several authors that different inositol phosphate isomers could be effectively produced by microbial phytases [6–8].

In previous studies, we isolated *Pantoea* sp. strain 3.5.1 with the high level of phytase activity from forest soil [9]. The *Pantoea* sp. 3.5.1 *agpP* gene encoding phytase was identified, and the corresponding protein was purified and characterized. We identified D/L-*myo*-inositol 1,2,4,5,6-pentakisphosphate as the final product of the enzymatic reaction by *Pantoea* sp. 3.5.1 phytase [9]. Here, we report the expression of phytase encoding gene *agpP* from *Pantoea* sp. 3.5.1 in non-conventional yeast *Yarrowia lipolytica*. Interest in non-conventional yeasts such as *Y. lipolytica* has increased over the years since these yeasts have been proven to be an attractive expression system for homologous and heterologous protein production due to the availability of genetic tools, characteristic, ability to utilize low-cost hydrophobic substrates, and excellent secretory capability [10]. It is "generally recognized as safe" (GRAS), status makes it especially a good candidate host for producing proteins used in pharmaceutical applications [11].

2 Materials and Methods

In this work, the gene sequence of histidine acid phytase from *Pantoea* sp. 3.5.1—*agpP* (AN KJ783401.1) was

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Fig. 1 Map of *agpP* expression constructs. Hp4d—*Y. lipolytica* hybrid promoter; XPR2—secretion signal peptide; His-tag—C-terminal 6 histidine tag; pBR-site—integration site

used. For cloning and expression of the phytase gene from *Pantoea* sp. 3.5.1 in *Y. lipolytica*, codon-optimization of *agpP* gene's nucleotide sequence was carried out using GenScript's OptimumGene™ Gene Design system (GenScript, USA). C-terminal His-tag was also added to the structural region of the gene. The codon-non-optimized native sequence of *agpP* gene has been deprived of its own signal peptide sequence, the C-terminal His-tag was added and was also used for cloning procedure. The optimized (*agpP-opt*) and natural (*agpP*) genes were amplified from the pUC57-agpP$_{opt}$ and pET28a-agpP plasmids. Both *agpP-opt* and *agpP* genes were cloned using KpnI and SfiI restriction sites into the pINA1296 vector. The resulting ligation constructs pINA1296/agpP$_{opt}$ (with codon-optimized phytase gene) and pINA1296/agpP (codon-non-optimized phytase gene without own SP) were transformed into *E. coli* DH5α cells. Transformants were analyzed by colony-PCR using primers to the yeast vector sequence and sequencing.

Resulting vectors pINA1296-agpP$_{opt}$ and pINA1296-agpP were linearized by SpeI restriction enzyme. *Y. lipolytica* strain Po1g was used for transformation by the Le Dall method [12]. Transformants were selected on the medium containing no leucine. Successful integration of the bacterial phytase genes into the *Y. lipolytica* genome was verified by genotyping and sequencing.

The expression of bacterial phytase in yeast culture media was detected by Western blotting and phytase activity assay. Western blotting with 6 His-tagged antibodies (Thermo Scientific) was carried out as described in protocol [13]. Phytase activity was quantified by a Greiner method measuring the amount of released inorganic phosphorous [14].

3 Results and Discussion

Codon optimization is widely used to enhance protein expression for heterologous expression. However, codon optimization does not always increase the level of heterologous protein expression [15]. Therefore, both codon-optimized and non-optimized genes were used for cloning; bacterial signal peptide was excluded from the sequence of non-optimized gene (Fig. 1). Yeast integrative vector pINA1296, which contains a strong hybrid promoter hp4d and a secretion signal (XPR2 pre region), was selected for cloning of bacterial phytase. Restricted vector and gene sequences were ligated. The

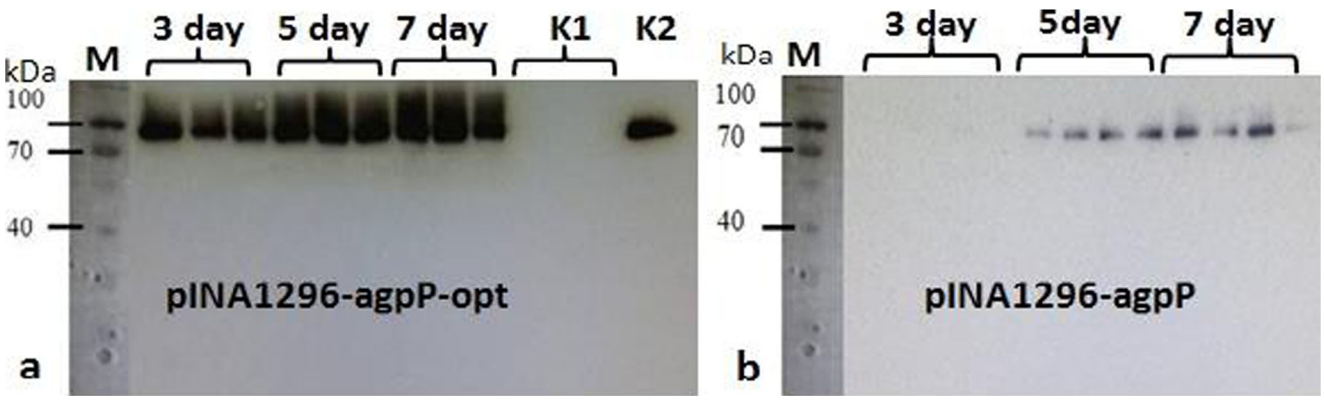

Fig. 2 Western blotting of culture liquid of recombinant *Yarrowia lipolytica* with pINA1296-agpP-opt constructs (**a**) and pINA1296-agpP constructs (**b**). M—marker, K1—untransformed *Y. lipolytica* strain (on 3, 5, and 7 days), K2—positive control (bacterial phytase AgpP expressed by *E. coli*)

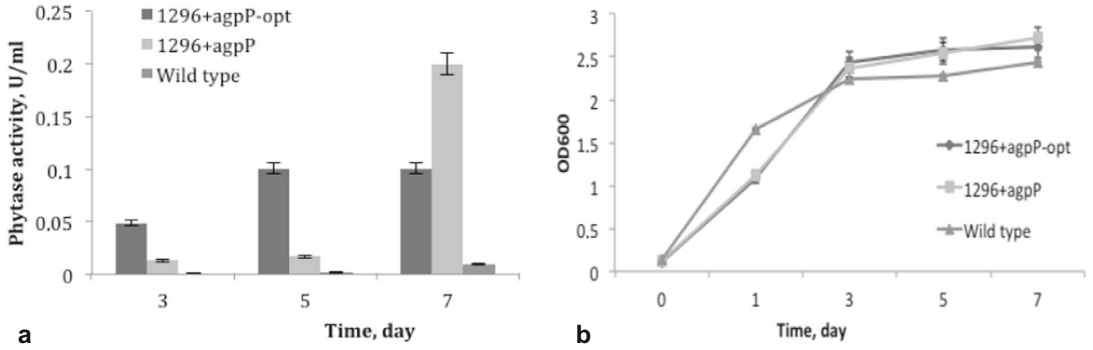

Fig. 3 Extracellular phytase activity (**a**) and growth curve (**b**) of the recombinant *Y. lipolytica*

received constructs pINA1296-agpP-opt and pINA1296-agpP, were transformed into *E. coli* DH5α. Genotyping confirmed the presence of PCR-products of about 1700 bp, which corresponds to the length of the *agpP* gene. Resulting recombinant plasmids pINA1296-agpP-opt and pINA1296-agpP were isolated, linearized, and transformed into *Y. lipolytica* Po1g cells. Integration of the bacterial phytase genes into the *Y. lipolytica* genome was confirmed by PCR analyses.

The ability of recombinant strains *Y. lipolytica* to express and secrete bacterial phytase in a culture medium was determined by Western blotting and phytase activity assay. XPR2 (encoding extracellular AEP protease) pre-region and terminator are most widely used for heterologous protein secretion. For the expression analysis, 4 recombinant yeast strains were selected from each of the constructs and grown on YPD medium. The hp4d promoter is constitutive; therefore, heterologous expression of bacterial phytase by yeast strains under the control of this promoter was performed using yeast culture fluid. Bacterial phytase AgpP expressed by *E. coli* with the molecular weight around 80 kDa was used as a positive control. Culture liquid of untransformed *Y. lipolytica* was used as a negative control. The Western blotting of the culture liquid of *Y. lipolytica* recombinant strains showed that a polypeptide with a molecular mass of about 90 kDa was produced. For the yeast strains *Y. lipolytica* pINA1296-agpP-opt and *Y. lipolytica* pINA1296-agpP expression was detected on the 3rd and 5th day of cultivation, respectively (Fig. 2). No intracellular phytase protein was detected by Western blotting in the recombinant strains.

In the culture medium of recombinant yeast, phytase activity was determined. For all transformants, phytase activity appeared on the 3rd day of cultivation and reached a maximum on the 7th day (Fig. 3a). Both *Y. lipolytica* recombinant strains reached the stationary phase of the growth by the 3rd day of cultivation, which maintained during the cultivation until day 7. The maximum phytase activity was possessed by the strain *Y. lipolytica* pINA1296-agpP; on the 7th day of cultivation, the activity reached 0.2 U/ml.

Thus, recombinant *Y. lipolytica* strains with integrated bacterial native (*agpP*) and optimized (*agpP-opt*) phytase genes were obtained. It was shown that bacterial phytase AgpP is expressed and secreted into the culture medium by recombinant yeast. Codon-optimization of phytase gene nucleotide sequence did not result in better production of recombinant phytase.

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