International Journal of Food Microbiology 205 (2015) 81–89

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Phytase-producing capacity of yeasts isolated from traditional African fermented food products and *PHYPk* gene expression of *Pichia kudriavzevii* strains

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ARTICLE INFO

Article history:
Received 1 December 2014
Received in revised form 19 March 2015
Accepted 6 April 2015
Available online 9 April 2015

Keywords:
Phytate
Phytase
RT-qPCR
Pichia kudriavzevii
African fermented food products

ABSTRACT

Phytate is known as a strong chelate of minerals causing their reduced uptake by the human intestine. Ninety-three yeast isolates from traditional African fermented food products, belonging to nine species (*Pichia kudriavzevii*, *Saccharomyces cerevisiae*, *Clavispora lusitaniae*, *Kluyveromyces marxianus*, *Millerozyma farinosa*, *Candida glabrata*, *Wickerhamomyces anomalus*, *Hanseniaspora guilliermondii* and *Debaryomyces nepalensis*) were screened for phytase production on solid and liquid media. 95% were able to grow in the presence of phytate as sole phosphate source, *P. kudriavzevii* being the best growing species. A phytase coding gene of *P. kudriavzevii* (*PHYPk*) was identified and its expression was studied during growth by RT-qPCR. The expression level of *PHYPk* was significantly higher in phytate-medium, compared to phosphate-medium. In phytate-medium expression was seen in the lag phase. Significant differences in gene expression were detected among the strains as well as between the media. A correlation was found between the *PHYPk* expression and phytase extracellular activity.

1. Introduction

Phytate is the salt of *myo*-inositol hexa*kis*phopshate (phytic acid, $InsP_6$), generally regarded as an anti-nutritional factor due to its ability to chelate cations such as Fe^{3+}, Zn^{2+}, Ca^{2+} and Mg^{2+}. Phytate-complexes are not available for absorption in the human intestine unless digested by phytases, a class of phosphatases that catalyze the hydrolysis of phosphate from phytate (Raboy, 2003).

Phytase is an enzyme of constantly growing attention, mostly as an animal but also human nutrition supplement. Phytases can be classified based on their active site motifs into histidine acid phosphatases (HAPs), β-propeller phytases (BPP) and purple acid phosphatases (PAP). HAP phytases are the most widely studied and they share a conserved N-terminal heptapeptide active site (RHGXRXP) and C-terminal catalytically active dipeptide, phosphohistidine (HD). The enzyme is naturally synthetized in plants and some microorganisms.

Yeasts have been reported as useful microorganisms for phytase production (Wykoff and O'Shea, 2001; Andlid et al., 2004; Nuobariene et al., 2011; Olstorpe et al., 2009; Sandberg and Andlid, 2002; Segueilha et al., 1992; Ushasree et al., 2014; Fonseca-Maldonado et al., 2014). The synthesis of phytases by yeasts is regulated by an external phosphate concentration and other factors such as: pH, temperature, carbon sources and occurrence of metal cations. The enzyme secretion can be intracellular, periplasmatic, directly to the culture medium or as bound to the cell wall. The genes encoding yeast phytases have so far been described for *Debaryomyces castelli* (*PHYDc*), *Kodamaea ohmeri* (*PHY1*), *Hansenula fabianii* (Hfphytase), *Pichia anomala* (*PPHY*), *Schwanniomyces occidentalis*, *Pichia stipitis* (*PHO5*), *Pichia guilliermondii* (*PGUG*), *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae* (*PHO3*, *PHO5*, *PHO11*, *PHO12*). In general, the identified ORFs contain the consensus motif (RHGXRXP) and encode a 451–467 amino acid protein.

Instead of using supplementation, fortification and synthetic addition of enzymes, phytate content in foods could be reduced by using high phytase-active microorganisms, in addition to food phytases (Fischer et al., 2014). Reduction of phytates by yeast phytases has been observed in a traditional food in Senegal (Antai and Nkwelang, 1999).

Pichia kudriavzevii (formerly known as *Candida krusei*) is reported to be involved in the fermentation of several traditional African foods (Greppi et al., 2013a,b; Jespersen et al., 1994, 2005; Pedersen et al., 2012). The species has also been reported to produce phytase (Nuobariene et al., 2011; Quan et al., 2002). Quan et al. (2002) have studied biochemical properties of a cell-bound phytase produced by *P. kudriavzevii* WZ-001. However, to our knowledge the gene encoding the *P. kudriavzevii* phytase has not been neither identified nor studied yet.

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.04.011
0168-1605/

In the present study we report on phytase-producing yeasts from traditional products commonly consumed in Benin (West Africa). The aims of the study were to identify the phytase-producing ability of autochthonous yeasts to improve our understanding about the phytase gene expression and enzymatic activity. Initially, several yeast strains were screened for phytase activity using growth-based liquid and solid tests. Subsequently, a phytase coding gene of *P. kudriavzevii* was identified and its level of expression in two growth media was investigated and compared with the corresponding enzyme activity measured during time.

2. Materials and methods

2.1. Yeast strains

Ninety-three yeast isolates from mawè, gowé, ogi and tchoukoutou, previously identified (Greppi et al., 2013a,b), were investigated in this study. They belong to nine species namely: *P. kudriavzevii* ($n = 44$), *S. cerevisiae* ($n = 20$), *Clavispora lusitaniae* ($n = 13$), *K. marxianus* ($n = 3$), *Millerozyma farinosa* ($n = 3$), *Candida glabrata* ($n = 3$), *Wickerhamomyces anomalus* ($n = 2$), *Hanseniaspora guilliermondii* ($n = 3$) and *Debaryomyces nepalensis* ($n = 2$). The isolate names were given as follows: the first letter represents the sample (M-G-O-T) followed by the progressive number of isolation. A genetically modified strain able to produce phytases constitutively was used as positive control (*S. cerevisiae* BY80, Euroscarf Acc. No. YO1692). All the isolates were stored at −80 °C in YPD medium (1% yeast extract (BD, Brøndby, Denmark), 2% glucose (Merk, Darmstadt, Germany), 2% bacto-peptone (BD), 15 g/l bacto agar (BD)) with 30% (*v*/*v*) glycerol.

2.2. Screening of phytase producing isolates

The ability of the isolates to grow in the presence of phytate as a unique phosphate source was tested on solid and liquid media as described by Nuobariene et al. (2011). Briefly, three different defined media were prepared: Delft Phy, a medium containing phytic acid dipotassium salt (1850 mg/l; Sigma P5681) as a unique phosphate source, Delft+, a medium containing phosphate (3510 mg/l) but no phytate, used as positive control, and Delft−, a phosphate-free medium, used as negative control. The protocols for preparation and composition of each medium were reported by Nuobariene et al. (2011). Few yeast colonies were inoculated in 10 ml sterile YPD medium and cultivated overnight in a water bath at 30 °C with shaking (170 rpm/min). Yeasts cultures, OD_{600} level set at 0.1, were transferred in 50 ml of YPD medium and cultivated for 10 h in a water bath at 30 °C with shaking (170 rpm/min). Subsequently, cells were spun down (Hermle Z216 MK, Germany) at 5000 ×*g* for 10 min, 4 °C, and the cell pellet was washed three times with 20 ml of sterile ultrapure water. The cell concentration was thereafter adjusted to an initial $OD_{600} = 1.0$. Two microliters of the 10^{-3} dilution was spotted in triplicates on Delft Phy, Delft+ and Delft− agar plates. The plates were incubated at 25 °C for 72 h and photographed afterwards. The growth was estimated by measuring the diameter of the colonies. Considering the same diameter range on Delft+ medium, results on Delft Phy plates were considered: negative (−), weak growth (+) when colony diameter was lower than 0.5 cm, normal growth (++) when diameter crossed 0.5 cm, high growth (+++) for all colonies reaching 1 cm and intense growth (++++) for colonies exceeding 1 cm. For liquid growth tests, 96-well microtiter plates (92096, TPP), wells were filled in triplicate with 200 μl of corresponding medium, inoculated with 2 μl of prepared yeast inocula and cultivated at 25 °C for 48 h. Yeast growth was monitored at OD_{600} using a Microplate reader (Varioskan Flash, Thermo Scientific, MA, USA). Measurements were taken every 2 h and 3 s shaking (300 rpm) was applied prior to data acquisition. For each medium, $\ln(OD_{tx} / OD_{t=0})$ for each time point was determined and standard deviation of three measurements was calculated. For each strain, OD_{600} measured at 48 h in Delft Phy was related to the OD_{600} in Delft+ medium. *S. cerevisiae* BY80 was used as positive control for all the growth-based tests.

2.3. P. kudriavzevii phytase gene expression

2.3.1. Yeast strains and growth conditions

Based on the results from the screening tests, five *P. kudriavzevii* strains were selected for further analysis. Four of them showed a high growth rate in Delft Phy (G6, G5, M31 and M30) while one failed to grow in Delft Phy medium (M16). Yeast inoculum was prepared as described above. Cells in exponential phase were used for inoculation of Delft Phy (100 ml) and Delft+ to an initial $OD_{600} = 1.0$. For extraction of total RNA, cells were spun down (1000 ×*g* for 1 min, 4 °C) at 0, 2, 4, 6, 8, 10, 14, 24 and 48 h. All pellets were frozen at −20 °C in the presence of RNA*later* (50 μl) and stored until RNA extraction.

2.3.2. RNA extraction, quantification and reverse transcription

Total RNA was isolated using Total RNA Mini Kit (Qiagen, Hilden, Germany) according to the supplier's manual. Cell lysis was performed by a step of bead-beating (3× 45 s, 4.5 m/s) in β-mercaptoethanol (600 μl) (Fast Prep, FP120 BIO 101, Savant, Santa Ana, CA, USA).

Table 1
Growth screening of 93 yeast strains in liquid Delft Phy (phytate-containing media) at 48 h. For each species, strains have been grouped according to the range of the actual OD_{600} values measured at 48 h in Delft Phy (max–min value). In the last column, the ratio OD_{600} Delft Phy/OD_{600} Delft+ (phosphate-containing media) is presented for each group as max–min value.

Yeast growth at 48 h in liquid Delft Phy medium					
Yeast species	No of strains (TOT)	No of strains (groups)	Strain ID	OD_{600} Delft Phy	OD_{600} Delft Phy/Delft+
Pichia kudriavzevii	44	43	G5, G6, G4, O13, T42, O12, O11, M32, M31, O10, M30, T41, M29, T40, T39, T38, G3, T37, M28, M27, M26, M25, T36, O9, T35, M24, M23, M22, M21, M20, O8, M19, T34, M18, T33, O7, O6, O5, T32, O4, O3, M17, T31	2.56–1.99	1.16–0.90
		1	M16	0.61	0.30
Saccharomyces cerevisiae	20	7	T30, T29, T28, T27, O2, T26, M15	2.51–2.01	1.01–0.94
		10	M14, T25, T24, M13, T23, T22, T21, T20, T19, T18	1.75–1.28	1.06–0.95
		3	T17, T16, T15	0.59–0.51	0.36–0.33
Wickerhamomyces anomalus	2	–	M4, M3	2.31–2.07	1.01
Millerozyma farinosa	3	–	M10, M9, M8	2.29–1.50	0.99–0.84
Candida glabrata	3	2	M7, M6	2.27–1.43	1.01–0.97
		1	M5	0.35	0.20
Kluyveromyces marxianus	3	–	M2, G1, M1	2.17–1.43	1.02–0.95
Clavispora lusitaniae	13	5	M12, G2, O1, T14, T13	2.12–1.70	1.03–0.96
		8	T12, T11, T10, T9, T8, T7, M11, T6	1.58–1.31	1.03–0.76
Hanseniaspora guilliermondii	3	–	T5, T4, T3	1.66–1.61	1.05–1.04
Debaryomyces nepalensis	2	–	T2, T1	1.15–1.11	0.96–0.89
Positive strain	1	–	BY80	2.50	1.00