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Phytase production by *Candida melibiosica* 2491 alkalophylic strain

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Abstract

It has been proved that *Candida melibiosica* 2491 yeast strain, an intracellular phytase producer, can be well adapted to a nutritious medium with a pH range from 3.0 to 9.0. The best development of the strain was achieved at pH 8.0, which refers to the rarely encountered alkalophylic yeast strains. The maximum biosynthesis was obtained in a medium with pH of 8.5, 24h-hour aged inoculum added at concentration of 6.6% (v/v) with optical density OD₆₀₀ of 0.68, aeration degree 500 dm³/dm³.h, stirring speed 300 rpm, and 24h duration of the cultivation in bioreactor. The optimal phytase activity was reached at 60°C and pH 4.5. However, over 80% of the maximum enzymatic activity was observed at the pH interval ranging from 4.0 to 8.5, which provides great potential for practical applications.

Key words: Alkalophylic strain, *Candida melibiosica*, Phytase activity

Introduction

Phytases (myo-inositolhexakisphosphate phosphohydrolases) are enzymes, which catalyze the stepwise decomposition of phytic acid (IP₆), resp. phytates, to inositol phosphate esters (IP₅–IP₁) and inorganic phosphate. Different classifications of phytases have been proposed: i) depending on the C-atom (dephosphorilation of myo-inositol ring is initiated from it) – 3-phytases (E.C. 3.1.3.8), 6-phytases (E.C. 3.1.3.26) and 5-phytases (E.C. 3.1.3.72); ii) according to their pH optima – acid and alkaline phytases iii) based on the catalytic mechanism - histidine acid phytases, β-propeller phytases, cysteine phytases and purple acid phytases (Mullaney and Ullah, 2003, Greiner and Konietzny, 2006).

There is a great potential for the use of phytases in the processing and manufacturing of forage or foods for human consumption (Haefner et al., 2005) as well as for environmental protection (Lei and Stahl, 2001). The pH stability of *Candida melibiosica* 2491 also determines the strains

possible application in bioremediation and biofuel cells (Hubenova and Mitov, 2011).

In both plant seeds and microorganisms, constitutive as well as inducible phytases have been identified. In non-limiting media, the majority of the bacterial phytases formation is turned off in exponentially growing cells and starts as soon as the cultures enter the stationary phase. In molds, the phytase formation was shown to be growth associated (Dvořáková, 1998; Konietzny and Greiner, 2002).

Phytase yeast producers receive more attention due to their easy handling and improved cultivation conditions, as well as due to their non-pathogenic features which allow their application in direct food supplement production. Data regarding phytases produced by yeast from genera *Schwanniomyces* (Lambrechts et al., 1992), *Saccharomyces* (Žyla, 1994), *Rhodotorula* (Bindu et al., 1998), *Arxula* (Sano et al., 1998), *Candida* (Quan et al., 2001), *Pichia* (Vohra and Satyanarayana, 2001), *Kodamaea* (Li et al., 2007), *Cryptococcus* (Pavlova et al., 2008) were reported. Each microorganism's required specific cultivation however necessitates studies aimed at improving the phytase expression and activity.

In our previous paper, we reported that *Candida melibiosica* 2491 yeast strain possesses the highest phytase activity among the one hundred and eighteen screened microorganisms (Georgiev

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and Gargova, 2006). The aim of this study is to optimize *Candida melibiosica* 2491 cultivation conditions as well as to establish enzyme optima of the phytase.

Materials and Methods

Yeast strain

The yeast strain *Candida melibiosica* 2491 is maintained on YPfru agar nutrition medium containing (in g/L): fructose – 30.0, yeast extract – 5.0, meat peptone – 8.0, and 0.2 mM KH₂PO₄. YPfru broth was used for the preparation of the inoculum.

Flask cultivation

A 30 mL mixture of yeast cell inoculum and liquid medium with the above mentioned composition was placed in 300 mL Erlenmeyer flask and put on a shaker (3.7 Hz) at 28°C (Georgiev and Gargova, 2007).

Optimization of cultivation conditions

In order to optimize the cultivation conditions, the experiments were carried out in broth media with different initial pH, and inoculum of different age and quantity was used. The nutritious media had initial pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. Inocula were taken at the 18th, 24th, 36th and 48th hours of cultivation, and their optical density was standardized to OD₆₀₀=0.68. From each sample, various concentrations between 1.65 and 10% (v/v) were used for further cultivation. At the 30th hour of the yeast development, the dry biomass (DW) and the phytase activity (PhA) were determined.

Bioreactor cultivation

In a 2L bioreactor MBR Mini, (AG, Switzerland) a periodic cultivation process was carried out, using 1L yeast cells/medium at initial pH 8.5. The pH, dry biomass, trace phosphorous (Pi) quantity, quantity of the reducing sugars and phytase activity were monitored during the process. The influence of the cultivation conditions, such as aeration degree (50 – 1000 dm³/dm³.h) and stirring speed (300 to 600 min⁻¹), was also studied.

Temperature and pH profiles of phytase activity

The influence of temperature on the hydrolysis of sodium phytate was examined at 30, 37, 45, 50, 55, 60, 65, 70 and 80 °C. The pH of the substrate solutions was adjusted accordingly by using the following buffers: 0.2 M glycine – HCl (pH 2.0, 2.5, 3.0, 3.5); 0.2 M sodium acetate – acetic acid (pH 4.0, 4.5, 5.0, 5.5, 6.0); 0.05 M Tris – HCl (pH 6.5, 7.0, 7.5, 8.0, 8.5), 0.05 M glycine – NaOH (pH 9.0, 9.5, 10.0).

Analytical methods

Yeast cells, centrifuged at 10000 rpm, were washed twice with 0.2 M sodium acetate buffer, pH 5.5, and then re-suspended in 5 mL of the same buffer (Žyla, 1994). The growth rate and the biomass accumulation were determined by means of optical density measurement (OD₆₀₀). The reducing sugars were quantitatively determined by means of 3,5 - DNS method (Miller, 1959).

The phytase activity was estimated from the quantity of the formed inorganic phosphate, determined by the Engelen's method (Engelen et al., 1994). The optical density of the color solution containing ammonium heptamolybdate (Scharlau, AM0349) and ammonium monovanadate (Scharlau, AM0467) was measured at 415 nm. One unit phytase activity is equivalent to the production of 1 μmol inorganic orthophosphate and is denoted as U/g dry biomass (Segueilha et al., 1992).

Statistical processing

The results are presented as their mean values and standard deviation of three independent experiments "Sigma Plot 11" software was used for presentation of the results and statistical analysis.

Results and Discussion

Flask cultivation

A good yeast cell development was observed in cultivation media with a wide range of the initial pH (Figure 1). The maximum growth, however, was achieved at pH 8.0, which determines *Candida melibiosica* 2491 as an alkalophylic strain. It should be noted that the strain also develops well at acidic pH from 3.0 to 5.5, which is an indication of the rich physiological potential of this microorganism. The enzymatic assays, however, show that phytase expression is observed at or above pH 5.0, and the maximum enzyme productivity was at pH 8.5. At this pH value, the highest yield factor (Y_{biomass/substrate}) of 42.6% was also obtained. In a similar study, Quan et al. (2001) established almost the same development of *Candida krusei* in the pH range from 4.0 to 9.0. However, the highest phytase activity was obtained at pH 7.0, and it was close to the maximum at pH 6.0 and pH 8.0.

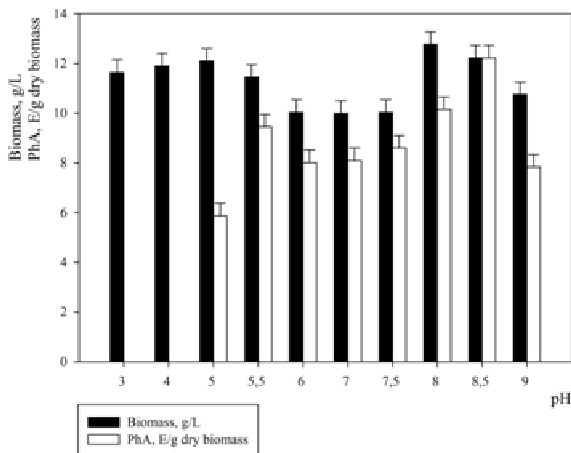


Figure 1. Influence of the medium's initial pH on the development of *Candida melibiosica* 2491 and phytase biosynthesis.

The inoculum age only slightly affects the culture development, but significantly influences the phytase productivity. Maximum enzyme activity was achieved when 24 h-aged inoculum was used (Figure 2a). The increase of inoculum quantity enhances the yeast biomass production. However, maximum phytase activity was observed when 6.6% inoculum was used (Figure 2b). During the optimization of the chemical composition of the nutrient medium was determined that 0.2 mM KH₂PO₄ stimulates the production of phytase. At higher Pi levels the phytase activity is inhibited, while lower Pi levels decrease but do not inhibit the enzyme production. The introduction of larger inoculum quantities probably leads to a decrease in the biomass/Pi proportion. Based on the obtained results, 24h-aged, 6.6% inoculum/media was chosen as optimal for *Candida melibiosica* 2491 cultivation and phytase production.

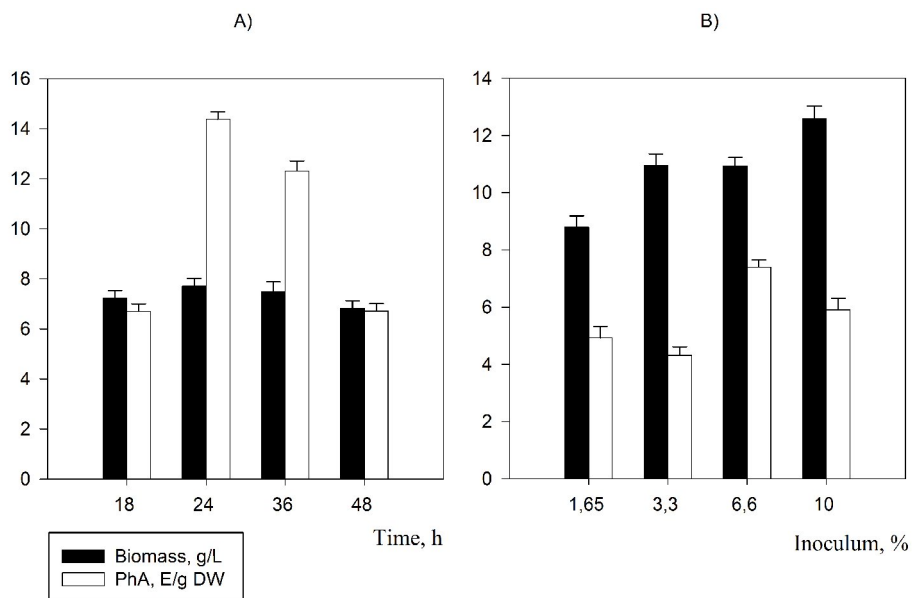


Figure 2. Influence of: a) the inoculum age and b) the inoculum quantity on the *Candida melibiosica* 2491 development and phytase productivity.

Bioreactor cultivation

The transition from flask cultivation to bioreactor cultivation is an important scale-up stage in the development of the biotechnological process for producing intracellular phytase from the yeast strain *Candida melibiosica* 2491.

After determining the optimal conditions in flask (pH of media, age and quantity of inoculum), cultivation of *Candida melibiosica* 2491 in a

bioreactor was performed. The influence of the aeration degree and stirring speed on the yeast growth and phytase activity was determined. The aeration degree α (dm³/dm³.h) was estimated by the equation $\alpha = Q/V$, where, Q is the air debit (dm³/h), V is the working volume (dm³).

The yeast biomass formation was enhanced by increasing the aeration degree. At low aeration degree ($\alpha = 50$ dm³/dm³.h), both the accumulated

biomass and enzyme activity were approximately at one half of the maximum values (Figure 3). The best development of the culture was achieved at $\alpha = 1000 \text{ dm}^3/\text{dm}^3 \cdot \text{h}$ (19.4 g/L biomass), even though at $\alpha = 500 \text{ dm}^3/\text{dm}^3 \cdot \text{h}$ the obtained biomass quantity was also very close to the maximum. The highest phytase activity of 13.2 E/g dry biomass was measured at $\alpha = 500 \text{ dm}^3/\text{dm}^3 \cdot \text{h}$, which determined this aeration degree as optimal for further experiments.

The variation of stirring speed from 300 to 600 min^{-1} did not affect significantly the cell development. However, it was an important factor for the phytase biosyntheses (Figure 4). The enzyme activity determined at 600 min^{-1} was almost two times lower than the maximum achieved at 300 min^{-1} (13.0 E/g dry biomass).

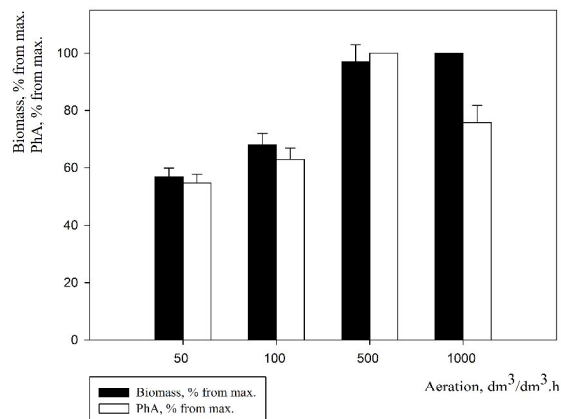


Figure 3. Influence of the aeration on *Candida melibiosica* 2491 development and enzyme activity in 2L bioreactor: pH 8.5; stirring rate 300 min^{-1} ; temperature 28°C; duration 24 h.

In the progress of the biosynthetic process, the main carbohydrate source fructose was intensively assimilated, and at the 12th hour residual sugars were not been measured (Figure 5). The total exhaustion of carbohydrates corresponds to the end of the exponential growth phase of *Candida melibiosica* 2491 and the maximal accumulation of biomass. The inorganic phosphate (Pi) in the medium rapidly decreased during the first 8 hours, and its complete depletion was achieved at the 18th hour of cultivation. The phytase formation continuously grew with the highest rate between the 8th and 18th hour of cultivation, corresponding to the exponential and early stationary phase of the culture development. pH of the cultural medium did not vary in a wide range – from the initial value of

8.5, it reached minimum of 7.5 at the 12th hour and after that increased to 8.0 at the end of the process. In their study Vohora et al. (2011) also investigated the process dynamics but in *Pichia anomala* (Vohora et al., 2011). For this train the carbon source quantity is 4%, the inoculum is 5% and the process duration is 24 hours.

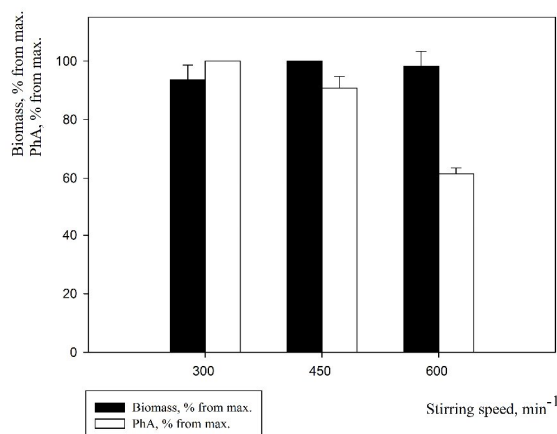


Figure 4. Influence of the stirring speed on *Candida melibiosica* 2491 development and enzyme activity in 2L bioreactor: pH 8.5; aeration degree 500 $\text{dm}^3/\text{dm}^3 \cdot \text{h}$; temperature 28°C; duration 24 h.

Enzyme optima

The temperature profile of the intercellular phytase activity showed a typical behavior – the activity gradually grew up to 60°C, then it sharply decreased and was totally lost at 80°C (Figure 6a). The observed profile is in agreement with other established data. However, the temperature optima for different phytase producers varies greatly: *Candida krusei* WZ – 001 – 40°C (Quan et al., 2001); *Pichia anomala* – 60°C (Vohra et al., 2001); *Schwanniomyces castellii* – 77°C (Segueilha et al., 1992); *Arxula adenivorans* – between 75°C and 82°C (Sano et al., 1998).

It was determined that the phytase produced by *Candida melibiosica* 2491 is active in a very wide pH range from 4.0 to 8.5 with pH optimum at 4.5 (Figure 6b). The activity at pH 4.0 is 88% and at pH 8.5 – 80% of the maximum. The most often reported yeast phytases also exhibit pH optima in the 4.0 – 5.0 pH interval: *P. anomala* – pH 4.0 (Vohra et al., 2001); *A. adenivorans* – pH 4.0 (Sano et al., 1998). An exception is the phytase from *P. pastoris*, which has a broad pH optimum at pH values from 2.0 – 5.5 (Yin et al., 2005).

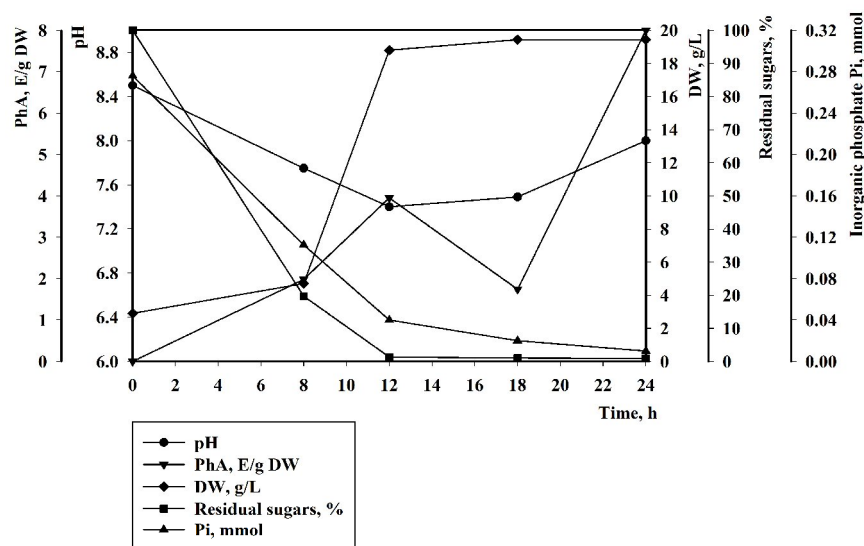


Figure 5. Dynamics of phytase biosynthesis by *Candida melibiosica* 2491, cultivated in 2L bioreactor: YPfru medium initial; pH 8.5; 6.6% inoculum; stirring rate 300 min⁻¹; aeration degree 500 dm³/dm³.h; temperature 28°C; duration 24 h.

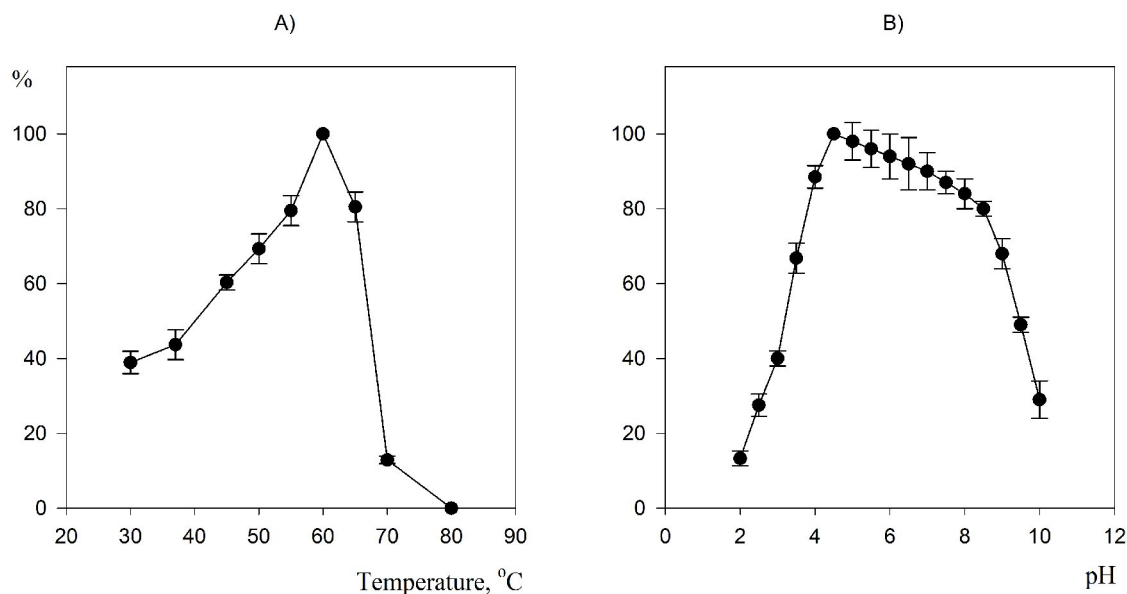


Figure 6. Influence of: a) the temperature and b) pH on the phytase activity.

The high phytase activity at different pH values - from acidic to weak alkaline range - reveals potential for applications of the investigated strain as a supplement for animal and human diets (Fu et al., 2008). As an enzyme active at acidic pH, the phytase is suitable for supplementing monogastric animals' diets. Acidic phytases might induce a more effective hydrolysis of phytate in both the stomach and small intestine of animals in terms of

the pH of the animal gastrointestinal tract (Park et al., 1999). They show broad substrate specificity and hydrolyze metal-free phytate at the acidic pH range, producing myo-inositol monophosphate as the final product. In contrast, the alkaline phytases exhibit strict substrate specificity for the calcium-phytate complex, existing in the animals' digestive tract, and produce myo-inositol trisphosphate as the final product (Oh et al., 2004).

Further studies regarding the biotechnological development of *Candida melibiosica* 2491 for animal nutrition and human health are currently being investigated.

Conclusions

It is proved that *Candida melibiosica* 2491 has an optimum activity for phytase production at pH 8.5. Based on the literature survey, this is the only strain with an alkaline pH optimum. Under flask cultivation, higher enzyme activity was achieved, which is due to the better control over the cultivation parameters, especially pH and aeration control. In addition, better heat and mass transfers were also observed. In the laboratory bioreactor, a higher amount of biomass was reached, and because of this the total amount of enzyme is higher. Although *Candida melibiosica* 2491 is an alcaliphilic strain, it exhibits great physiological potential regarding the initial pH values. The high protein content of the strain combined with its phytase activity will improve the quality of feed by its introduction in industrial production.

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