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Investigation of the potential of yeast strains for phytase biosynthesis in a two-step screening procedure

Danail Georgiev [a], Milena Kostova [b,*], Ana Caroline de Oliveira [c], Yordan Muhovski [c]

[a] University of Plovdiv, Faculty of Biology, Department of Biochemistry and Microbiology, 24 Tsar Ivan Asen Str., Plovdiv 4000, Bulgaria
[b] Agricultural University – Plovdiv, Faculty of Agronomy, Department of Plant physiology, Biochemistry, and Genetics, 12 Mendeleev blvd., Plovdiv 4000, Bulgaria
[c] Department of Life Sciences, Biological Engineering Unit, Walloon Agricultural Research Centre, 234 Chaussée de Charleroi, Gembloux 5030, Belgium

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ABSTRACT

Research into phytase production is useful for improving the efficiency of animal production, reducing environmental impact, and contributing to the development of sustainable and efficient animal production systems. This study aims to investigate the potential of yeast strains for phytase biosynthesis in nutrient media. Phytase is a phosphomonoesterase (E.C 3.1.3.8) catalyzing in a ladder-like manner the dephosphorylation of phytic acid and its salts, with various resulting myo-inositol phosphates and phosphoric acid. Yeasts of the genera *Saccharomyces*, *Zygosaccharomyces*, *Candida*, and *Pichia* were evaluated in a two-step screening procedure for phytase production. One hundred and eighteen strains were screened in the first stage, which was conducted on four types of solid culture media containing calcium phytate as the selected background. On PSM medium, many strains were found to form halos as early as the 24th hour of development. Several strains with significant potential for enzyme production were evaluated in the second step of the screening. It was conducted in a liquid culture medium. In conclusion, the strain *C. melibiosica* 2491 was selected for further studies when cultured in a YPglu culture medium. Further research will focus on finding suitable conditions that increase the biosynthesis of the enzyme, which is of significant technological and practical interest for animal nutrition.

1. Introduction

Due to the high demand, phytases have undergone extensive research to reduce the amount of phytates in human and animal foods (Rizwanuddin et al., 2023). Phytases (myo-inositol hexakisphosphate 3- and 6-phosphohydrolases; EC 3.1.3.8 and EC 3.1.3.26) are a subfamily of acidic histidine phosphatases that hydrolyze phytic acid with the release of one or more phosphoacid groups. In mature seeds of monocotyledonous and dicotyledonous plants, phytic acid is the major storage form of phosphorus, accounting for 60–90% of all organic phosphorus. Because of its strong chelating ability, it is considered an anti-nutritional factor as it forms insoluble complexes with nutritionally important metal ions such as calcium, zinc, magnesium, iron, etc., reducing their bioavailability and absorption. The organically bound phosphorus in phytic acid is not absorbed by monogastric animals such as pigs, poultry, fish and man due to the lack of the enzyme phytase, resulting in phosphorus contamination in areas of intensive livestock production. Therefore, reducing the phytic acid content of cereals by enzymatic hydrolysis will increase their bioavailability. Knowledge of the presence of high potential phytase-producing yeast strains can be a starting point for the development of different methodologies applied in the feed and food industry (Nuobariene et al., 2011; Qvirist et al., 2017).

Phytases are synthesized by microorganisms belonging to: *Aerobacter* (Greaves et al., 1967), *Bacillus* (Kerovuo et al., 1998), *Enterobacter* (Yoon et al., 1996), *Klebsiella* (Wang et al., 2004); mold fungi of the genus *Aspergillus* (Ullah and Cummins, 1988) and yeasts of the genera *Arxula* (Quan et al., 2001), *Candida*, *Clavispora*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Metchnikowia*, *Torulaspora* (Nakamura et al., 2000), *Schwanniomyces*, (Lambrechts et al., 1992), *Saccharomyces* (Nayini and Markakis, 1983), *Pichia*, *Rhodotorula* (Bindu et al., 1998). One of the tested and selected strains *Candida melibiosica* 2491 is famous for its exoelectrogenic properties (Hubenova, 2018). Metabolic pathways and mechanisms of extracellular electron transfer have been established (Hubenova et al., 2017; Hubenova and Mitov, 2015). Phytate hydrolysis was evaluated positively for *Saccharomyces cerevisiae* var. *boulardii*, which is important for increasing the functional value of plant-based food products (Menezes et al., 2020). In recent years, interest in yeast phytase has been growing, as evidenced by the increasing number of

* Corresponding author.
E-mail address: milena.kostova@gmail.com (M. Kostova).

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publications. The aim of the present study is to investigate yeasts of different genera and species for the selection of phytase enzyme producers.

2. Materials and methods

2.1. Microorganisms

The present investigations were carried out with 118 collection cultures of yeasts (listed in a supplementary Table S1), kindly provided by the LPBT of the Institute of Biology Plovdiv, Bulgaria), belonging to different genera, namely: 113 strains of the genus *Saccharomyces*, of which 63 - *S. cerevisiae*, 50 - *S. ellipsoideus*, 3 - of the genus *Candida*, of which 2 - *Candida tropicalis* and 1 - *Candida melibiosica*, 1 - of the genus *Zygosaccharomyces* and 1 species of the genus *Pichia*.

2.2. Nutrition media

Accessions are stored on APM (Agar beer must, Glucose 20 g/L, Agar 15 g/L, pH 6.6) solid medium at 0–4 °C and maintained by monthly inoculation.

Strains were cultured in 11 types of culture media, 8 of which have been cited by various authors in studies on the phytase activity of microorganisms from different systematic groups (Table 1). The remaining 3 types of media were modified after preliminary studies (Stanchev et al., 2010) and demonstration of the need for a corresponding change in chemical composition and pH values (Table 2). Both liquid and solid culture media were used to study secreted and intracellular phytase.

2.2.1. Qualitative test of yeast strains from different genera for phytase biosynthesis

Primary screening was performed by plating the strains on five types of solid culture media: PSM, MSAglu, MSAgal, YP, and PSM, in which calcium phytate served as a selective background for the detection of extracellular phytase activity. A method described by Engelen et al., 1994 was used. Standard phytase activity was determined relative to phosphate on 3 different days with 2 substrate weighings per day, 2 standard substrate weighings, and 2 weighing determinations. The results were statistically verified and averaged into activity. A microbial needle was used to seed a suture at no more than 5 points in the medium scattered on Petri dishes. The plates were incubated for 48 h at 28 °C. In the presence of phytase activity, halos appeared around the germinated colonies due to hydrolysis of the calcium phytate. The diameter of the halos is not an accurate indicator of the amount of phytase released.

Table 2
Chemical composition of modified culture media for phytase production.

Ingredients	Composition, g/L		
	9	10	11
	YP	PSM	YP_{fru}
Glucose	–	–	–
Fructose	–	–	30.0
Yeast extract	30	–	50
Malt extract	30	–	–
Peptone	5	–	80
Calcium phytate	5.0	5.0	–
KH_2PO_4	–	–	0.2 mM
$MgSO_4.7H_2O$	–	0.5	–
KCl	–	0.5	–
$MnSO_4.4H_2O$	–	0.01	–
$FeSO_4.7H_2O$	–	0.01	–
NH_4NO_3	–	5.0	–
pH	5.5	5.5	8.5

For this reason, the test is only used for qualitative but not quantitative analysis. Secondary screening was conducted in three stages. In the first stage, yeast were statically cultured in 2 mL tubes of YPglu liquid culture medium according to the method of Sano et al. (1999), and intracellular and extracellular activities were determined. The second step was also conducted in 10 mL of YPglu liquid culture medium poured into 250 mL Erlenmeyer flasks by shaking the apparatus culture at 28 °C for 48 h. The cultures were centrifuged at 10,000 rpm for 10 min. The supernatant was used to determine extracellular phytase activity, and cells washed twice with sodium acetate buffer (pH 5.5), were used to determine intracellular phytase activity according to the method of Żyła (1994), which uses whole cells.

In the next stage of the study, the four most active strains were cultured in flasks with four types of liquid culture media YPglu, YPgal, MSA and YEPD under the conditions described above.

2.3. Cultivation conditions of Candida melibiosica 2491 on YPfru media for phytase production

Studies were conducted with *Candida melibiosica* 2491 on modified YPfru low phosphate content (less than 0.1 mM) agar medium containing (g/L): yeast extract - 50, meat peptone - 80 and fructose - 30.0 (medium 11, Table 2). The available phosphate in the basal medium (about 3 mM) was precipitated by Sano et al. (1999) by adding 10 mM $MgCl_2$ and adjusting the pH to 8.6. After a 24 h residence time in ice, the precipitated phosphate was separated by filtration.

Table 1
Chemical composition of culture media for primary screening of yeasts for phytase production.

Ingredients	Composition, g/L							
	1	2	3	4	5	6	7	8
	YPglu (Sano et al., 1999)	YPgal (Sano et al., 1999	YPD (Yin et al., 2010)	YEPD (Żyła, 1994)	PSM (Howson and Davis, 1983)	MSAglu (Lambrechts et al., 1992)	MSAgal (Lambrechts et al., 1992)	Quan (Quan et al., 2001)
Glucose	20.0	–	20.0	50.0	15.0	10.0	–	50.0
Yeast extract	10.0	10.0	10.0	30.0	–	–	–	–
Peptone	10.0	10.0	20.0	10.0	–	–	–	7.0
Galactose	–	20.0	–	–	–	–	10.0	–
$MgSO_4.7H_2O$	–	–	–	–	0.5	0.5	0.5	5 mM
KCl	–	–	–	–	0.5	0.5	0.5	10 mM
$CaCl_2$	–	–	–	–	–	0.1	0.1	–
$MnSO_4.4H_2O$	–	–	–	–	0.01	0.0079	0.0079	–
Calcium phytate	–	–	–	–	5.0	2.0	2.0	–
KH_2PO_4	–	–	–	–	–	–	–	0.02
$MnCl_2.4H_2O$	–	–	–	–	–	–	–	1 mM
$FeSO_4.7H_2O$	–	–	–	–	0.01	–	–	0.1 mM
$(NH_4)_2SO_4$	–	–	–	–	–	3.0	3.0	–
NH_4NO_3	–	–	–	–	5.0	–	–	–

Depth culturing was performed into 300-mL Erlenmeyer flasks with liquid YPfru medium at 10% fill volume. Seeding was performed with 6.6% (*v*/v) inoculum from 24 h old culture and with the absorbance of 0.68 measured at 600 nm. The process was carried out on a shaker at 28 °C and 220 rpm for 30 h.

3. Results and discussion

3.1. Screening of yeast strains for phytase production

Reliable screening methods have been reported to elucidate the ability of different yeast strains to utilize phytic acid as the sole source of phosphorus (Olstorpe et al., 2009; Capusoni et al., 2021). Furthermore, a link between exoelectrogenicity and phytase production has also been established (Hubenova et al., 2014; Hubenova et al., 2014). The key steps in phytase production are the selection of culture parameters, followed by the isolation of the enzyme and the evaluation of catalytic activity (Kłosowski et al., 2018). The screening of phytase producers involves two steps: primary and secondary. The primary stage is performed qualitatively by a method previously described by (Howson and Davis, 1983), which uses a solid phytase screening medium (PSM) containing glucose as a carbon source and calcium phytate as a selection background that imparts turbidity to the medium. A rapid method, based on the determination of inorganic orthophosphate released by hydrolysis of sodium phytate to determine the enzymatic activity of microbial phytase, has been described previously (Engelen et al., 1994). The yeast strain *Pichia pastoris* has recently been engineered to express and secrete phytase in response to an external level of inorganic phosphate (Pi) (Xie et al., 2020). Since our initial idea and expectation was to work with yeast extracellular phytase producers, all producers were subjected to primary screening. Achievement of high extracellular phytase activity by the yeast *P. kudriavzevii* TY13 by regulating growth medium phosphate concentrations has also been reported (Hellström et al., 2015). In the presence of extracellular phytase activity, halos occur around the colonies due to hydrolysis of the calcium phytate and its conversion to a form digestible by the yeast cells. Secondary selection involves the application of quantitative methods in which the exact values of both extracellular and intracellular phytase activity are established.

3.2. Primary screening

On PSM (containing glucose as a carbon source) and MSAgal (containing galactose as a carbon source), almost all yeast cultures examined showed halos as early as the 24th hour (Fig. 1). On YP agar medium, which does not contain a readily digestible carbon source, only 10 strains of *Saccharomyces cerevisiae* showed halos. No growth of microorganisms was observed on PSM synthetic medium, which does not contain a carbon source. The reason was its poor chemical composition.

The halo diameters of all 118 tested strains cultured on two types of solid culture media, PSM and MSAgal, containing 5.0 and 3.0 g/L calcium phytate, respectively, as selection background (Table S1). Nine strains of the first species and two strains of the second species had the highest enzyme activity with MSA halos greater than 17 mm. The result is consistent with the data of studies in which the applied $CaCl_2$ concentration has a place on the increase of phytase activity and related gene expression (Yan et al., 2017).

On PSM medium, only one strain of *S. cerevisiae* showed zero phytase activity, while eight strains of the same species and strain *Zygosaccharomyces marxianus* 1562 had a halos with diameter of up to 10 mm. The highest activity was shown by 13 strains of *S. cerevisiae* and 2 strains of *S. ellipsoideus*. Regarding the duration of cultivation, there was an almost proportional increase in the halos diameter with increasing cultivation time from the 24th to the 72nd hour in all cultures. Since many microorganisms produce acids that lower the pH value around the colonies, the result is that sufficient phosphorus is released for their growth due to the acid hydrolysis of the calcium phytate.

3.3. Secondary screening

After the primary screening, at the secondary screening stage, the strains were cultured in a liquid culture medium only. In the first stage of secondary selection, 20 strains that showed halos with the largest diameter in the primary screening were subjected to testing for intracellular and extracellular phytase activity: 12 from *S. cerevisiae*, 5 from *S. ellipsoideus*, 2 from *Candida* and 1 from *P. alcocholophyla*. The reported extracellular activity (U/L) of all strains tested was extremely low, while the intracellular activity was detected for strains *S. ellipsoideus* 2133, *C. melibiosica* 2491 and *P. alcchlophyla* 3612. Therefore, these microorganisms are not of interest for further investigation.

Exceptions are *S. ellipsoideus* 2133, with intracellular activity of 0.6 U/g ADB, and *C. melibiosica* 2491, with the highest intracellular enzyme activity of 2.06 U/g ADB and the highest total activity of 48.81 U/L. It should be noted that all strains studied developed well and biomass yield ranged from 8.03 to 9.23 g/L.

In a similar study, Lambrechts et al. (1992) investigated 21 yeast strains of 10 species, which *Schwanniomyces castellii* CBS 2863 developed on a medium with sodium phytate as the only phosphate source showed an enzyme activity 2.8 times higher compared to the other strains.

In the next stage of secondary screening, those four strains that showed the highest total enzyme activity on PSM medium were screened by culturing four more types of culture media: YPglu, YPgal, MSA and YEPD. As it is shown on Figs. 2 and 3, the selected cultures in some cases

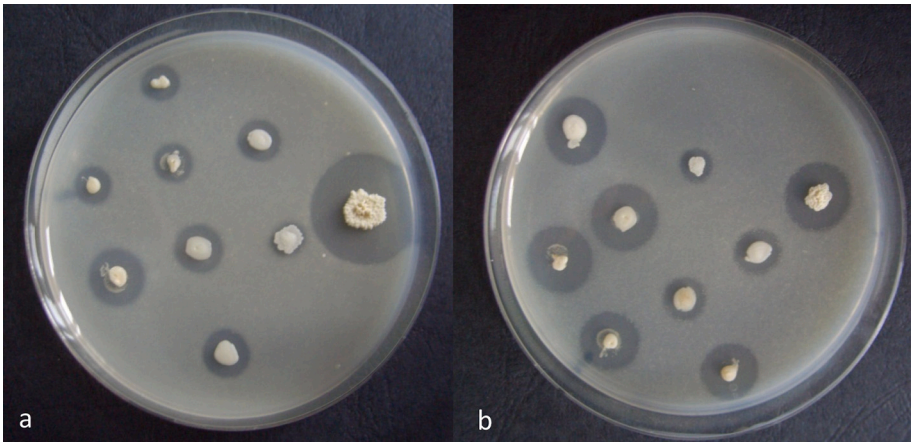

Fig. 1. Development and halos of yeast strains on two selective solid culture media: a) *S. ellipsoideus* 2133 on PSM, b) *C. melibiosica* 2491 on MSA.

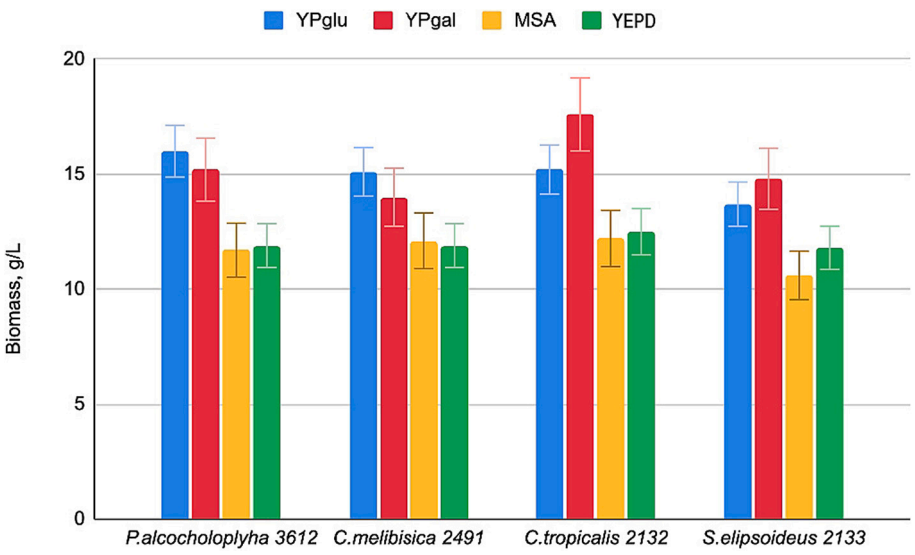

Fig. 2. Biomass of the most active strains of four types of liquid culture media.

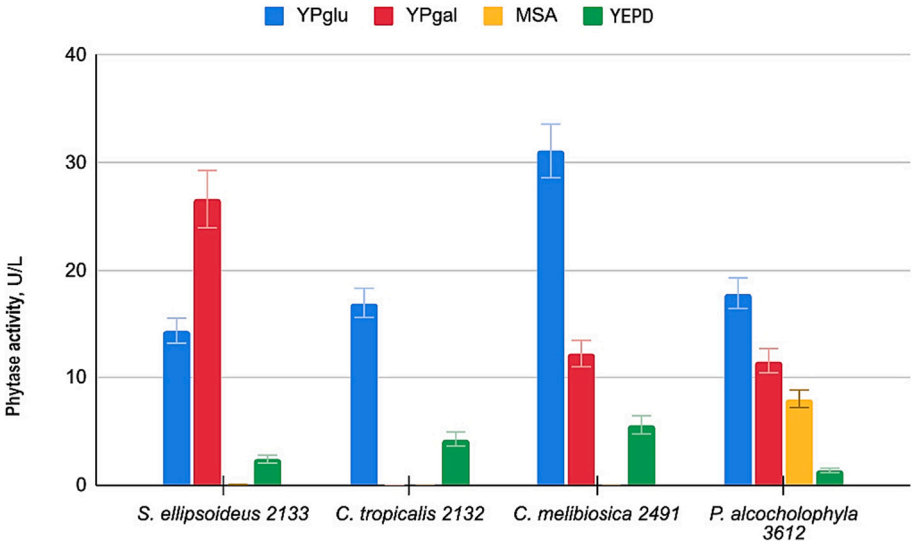

Fig. 3. Intracellular phytase activity of the most active strains of four types of liquid culture media.

exhibited very different development on the tested media. The differences in biomass yield were up to 44%. For the strains *S. ellipsoideus* 2133 on MSA and *C. tropicalis* 2132 the most suitable medium was YPgal, with biomass yields of 14.8 and 17.6 g/L, respectively. Compared to the PSM medium, the increases were 43.7 and 41.9%, respectively. The YPglu medium proved to be the most suitable for the strains *C. melibiosica* 2491 and *P. alcocholophyla* 3612, which showed biomass increases of 15.1 and 16.0 g/L, respectively (Table 3). These results represent 22.8 and 33.3% higher growth compared to the PSM medium. The same was observed for intracellular phytase activity. The most important index of enzyme production per unit volume of culture fluid, U/L, was quite different among the phytase-producing yeasts cultured in the four culture media (Table 4).

As a result of the selection of phytase-producing yeasts, it was found that the highest phytase activity was exhibited by *C. melibiosica* 2491 and *P. alcocholophyla* 3612 on YPglu medium.

Two strains, *C. melibiosica* 2491 and *P. alcocholophyla* 3612, were tested in the next stage of secondary screening as promising intracellular phytase producers. The effect of five other types of liquid culture media, including YPD, YEPD, MSAglu, MSAgal and Quan, recommended by various authors (Lambrechts et al., 1992; (Żyła, 1994; Quan et al., 2001), was investigated. Enzyme production was compared with that obtained on PSM medium serving as control. The results are presented in Table 5. For strain *P. alcocholophyla* 3612, YEPD and Quan's medium were the most suitable for development (biomass growth 8.53 and 8.54 g/L, respectively). However, enzyme production was much lower in the former medium (4.95 U/L), whereas it was zero in the Quan medium. For strain *C. melibiosica* 2491 the most suitable medium for biomass accumulation was YPglu (Absolute Dry Biomass ADB 14.8 g/L). Phytase activity was also highest at 2.06 U/g ASB. Logically, enzyme production was also maximum 30.49 U/L on this medium. The Quan medium was also suitable with a biomass yield of 10.23 g/L. In comparison, the YEPD medium accumulated a significant amount of biomass, 13.23 g/L, but since the phytase activity was twice lower (0.96 U/g ASB), the enzyme production (12.70 U/L) was 58.3% lower compared to the YPglu medium.

Based on the results obtained in the following studies, *C. melibiosica* 2491 was selected as the most efficient producer when cultured in YPglu medium.

Based on preliminary studies, the strain *C. melibiosica* 2491 was the

Table 3
Activity of 20 phytase-producer yeast strains cultured in YPglu medium during 48 h.

Strain	Absolute dry biomass (ADB*), g/L	Extracellular activity, U/L	Intracellular activity, U/g ADB
S. cerevisiae 3132	8.35	31	0
S. cerevisiae 2411	8.7	18	0
S. cerevisiae 2145	8.03	20	0
S. cerevisiae 2233	8.77	22	0
S. cerevisiae 2236	8.53	23	0
S. cerevisiae 2238	8.75	33	0
S. cerevisiae 2401	8.42	26	0
S. cerevisiae 2454	8.23	29	0
S. cerevisiae 2455	8.55	30	0
S. cerevisiae 2460	8.14	30	0
S. cerevisiae 2408	8.34	32	0
S. cerevisiae 2158	8.35	22	0
S. ellipsoideus 2213	9.01	22	0
S. ellipsoideus 2133	9.15	31	0.6
S. ellipsoideus 2315	9.23	28	0
S. ellipsoideus 2422	9.2	32	0
S. ellipsoideus 2454	9.06	33	0
C. tropicalis 3132	9.12	36	0
C. melibiosica 2491	9.13	30	2.06
P. alcocholophyla 3612	8.33	35	0.38

* ADB - Absolute Dry Biomass.

Table 4
Production of intracellular phytase by four yeast strains cultured in four types of culture media.

	S. ellipsoideus 2133, U/L	*C. tropicalis* 2132, U/L	*C. melibiosica* 2491, U/L	*P. alcocholophyla* 3612, U/L
YPglu	14.45	17.02	31.11	17.92
YPgal	26.64	0	12.32	11.62
MSA	0.1	0	0	8.04
YEPD	2.44	4.31	5.62	1.4

Table 5
Biomass growth and phytase activity of *C. melibiosica* 2491 and *P. alcocholophyla* 3612 in liquid culture media.

Nutrition media	*P. alcocholophyla* 3612			*C. melibiosica* 2491		
	ADB, g/L	PhA, U/g ADB	Production, U/L	ADB, g/L	PhA, U/g ADB	Production, U/L
YPglu	8.33	0.38	3.17	14.8	2.06	30.49
YPgal	5.34	0.88	4.71	8.47	0.90	7.62
YPD	8.02	0.70	5.61	9.81	0.50	4.91
YEPD	8.53	0.58	4.95	13.23	0.96	12.70
PSM	4.51	0	0	6.12	0	0
MSAglu	5.88	0	0	5.70	0	0
MSAgal	4.73	0	0	8.16	0.62	5.06
Quan	8.54	0	0	10.23	0.42	4.30

ADB - absolute dry biomass.
PhA – phytase activity.

most productive in terms of the target enzyme and *P. alcocholophyla* 3612 was the least demanding in terms of culture conditions and medium composition.

P. alcocholophyla 3612 grew very well on four types of culture media including Quan, YEPD, YPglu and YPD, accumulating almost the same amount of biomass, 8.54 and 8.02 g/L. The strain *C. melibiosica* 2491 grew optimally on YEPD medium, with a biomass yield of 13.23 g/L, 55% higher than the maximum yield of *P. alcocholophyla* 3612. Relatively good growth was recorded on Quan (10.23 g/L) and YPD (9.81 g/L) media. The biomass yield characteristic did not correlate with phytase activity. The two selected producers exhibited strain specificity: for *P. alcocholophyla* strain 3612, the most suitable media for activity were YPgal and YPD, using which the amount of enzyme synthesized was 0.88 and 0.70 U/g ADB, respectively; the media PSM, MSAglu and MSAgal and Quan were unsuitable: zero activity was recorded.

The highest enzyme production was observed when YPD was used - 5.61 U/L. For strain *C. melibiosica* 2491 the most suitable medium was YPglu, in which a maximum activity of 2.06 U/g ADB was recorded, i.e. 2.3 times higher than the highest activity for strain *P. alcocholophyla* 3612. The results are consistent with those previously presented by the research team (Georgiev et al., 2013; Georgiev et al., 2018). Zero enzyme activity was reported on PSM and MSAglu media.

4. Conclusion

Further research will focus on finding suitable conditions to increase the biosynthesis of the enzyme, which is of significant technological and practical interest for animal nutrition. Based on the results obtained from primary and secondary screening of phytase-producing yeasts using eight types of liquid culture media recommended in the literature as suitable for phytase biosynthesis, the strain *C. melibiosica* 2491 was selected for further studies. These results are particularly important for future research in the field of bioremediation of phytate-contaminated soil, animal nutrition, food processing, and more recently in the nutraceutical category.

Declaration of generative AI and AI assisted technologies in the writing process

No generative AI and AI-assisted technologies were used in the writing process of this paper.

Ethics statement

Not applicable.

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CRediT authorship contribution statement

Danail Georgiev: Investigation, Methodology, Writing – original draft. **Milena Kostova:** Data curation, Visualization, Writing – review & editing. **Ana Caroline de Oliveira:** Validation, Visualization, Writing – review & editing. **Yordan Muhovski:** Data curation, Supervision, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2024.106890.

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