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Phytase Production Using Citric Pulp and Other Residues of the Agroindustry in SSF by Fungal Isolates

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Summary

Phytases have important applications in human and animal nutrition because they hydrolyze the phytate present in legumes, cereal grains and oil seeds. This results in an increased availability of minerals, trace elements and amino acids as well as phosphate. Fifty potential phytase-producing fungal strains were isolated from a fertile soil obtained from the northern part of Paraná State in Brazil and other alternative sources using a selective media. Thereafter phytase production was evaluated in solid-state fermentation using different residues from the agroindustry supplemented with a nitrogen source at 60 % of moisture after 96 hours at 30 °C. The highest phytase activity (51.53 units per gram of dry substrate, U/g) was achieved with citric pulp and the soil isolate FS3 in solid-state fermentation. Furthermore, treatment of the substrates prior to fermentation in order to reduce microbial contamination was shown to affect phytase production during solid-state fermentation. Heat treatment resulted in an increase of the concentration of inorganic phosphate, a well known repressor of microbial phytase production, and therefore in a reduction of phytase production. UV exposure of the substrate was shown to reduce microbial contamination without affecting phytase production.

Key words: phytase, fungal strains, soil isolation, solid-state fermentation, citric pulp, phytate, agroindustrial residues

Introduction

Citric pulp (CP) is a solid residue resulting from the processing of oranges. Products derived thereof include dehydrated feed, pectin for food processing, citric acid, essential oils, molasses, and candied peel (*1*). Once the juice has been extracted from the oranges, peels and rags are left behind. They are equivalent to approx. 50 % of the processed fruit mass and are converted into CP pellets to be used in cattle feed (*2*). Martinez (*3*) reported that partial substitution of finely ground corn with pelletized CP does not affect milk yield. With a moisture level of about 12 %, CP was suggested to be an economic alternative substrate for enzyme production by solid-state fermentation.

Phytates (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) are salts of phytic acid and the major form of phosphorus is stored in seeds, fruits and legumes (*4,5*). Considerable amounts of phytate can be found in plant-based food products such as rice bran, oat flour, barley flour, wheat bran, beans, sesame bran, sunflower meal, soybean, cowpea, and sorghum (*6–10*). Phytate is also the most frequent form of phosphorus in soil (*11*). It chelates

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vital ions, thereby reducing their solubility and bioavailability. Phytate also forms complexes with proteins, which might result in an inhibition of digestive enzymes. Thus, high intakes of phytate result in poor utilization of nutrients. Therefore, phytate is considered to be an anti-nutritional factor (*12–15*). Moreover, monogastric animals are unable to use phytate phosphorus, because they lack endogenous phytase activity in their digestive tracts (*16,17*). To make the phytate phosphorus in the feed available to the animal and to reduce the addition of inorganic phosphorus to the feed in order to meet the animal's requirement for phosphorus, the use of phytases as feed supplement has steadily increased. Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are enzymes capable of initiating the hydrolysis of phytate resulting in partially phosphorylated *myo*-inositol phosphates and inorganic phosphate (*4,18,19*). The use of filamentous fungi for the phytase production through solid-state fermentation has gained much interest for research in the last years (*20*). The objective of this study is to compare phytase production in fungi isolated from soil and other habitats and to optimize phytase production during solid-state fermentation (SSF).

Materials and Methods

Isolation of microorganisms

Soil, corn, CP pellets, wheat bran and rice bran were used as a source material to isolate fungi. One gram of each source material was suspended in 100 mL of 0.1 % (by mass per volume) peptone water. The suspensions were incubated aerobically at 120 rpm on a rotary shaker at 30 °C for 24 hours. Then, 0.1 mL of a 10^{-2} to 10^{-8} dilution of the liquid medium in 0.1 % of peptone water was plated onto a solid phytate agar medium containing (in g/L): phytate (Sigma) 10, $NaNO_3$ 2, NH_4NO_3 2, $MgSO_4 \cdot 7H_2O$ 0.5, KCL 0.5, $ZnSO_4 \cdot 7H_2O$ 0.1, $FeSO_4$ 0.05, agar 19 and gentamicin sulphate (Shering-Plough) as described by Vats and Banerjee (*21*). The plates were incubated at 30 °C for 5 days. To isolate pure microorganisms, single colonies were transferred to potato dextrose agar (PDA) plates and the plates were incubated at 30 °C for 7 days. Thereafter, the plates were stored at 4 °C until use.

Inoculum preparation

Inoculum was prepared by suspending the spores present on the PDA agar plates in 20 mL of 0.1 % Tween 80. The number of spores was determined in a Neubauer counting chamber and the inoculum of 10^7 spores/g CP was used for SSF.

Study of different substrates in SSF

Different solid substrates like citric pulp (CP), apple pulp cv. Gala (APG), soy bran (SB), wheat bran (WB), rice bran (RB), citric pulp+apple pulp cv. Gala (CP+APG), citric pulp+soy bran (CP+SB), citric pulp+wheat bran (CP+WB), citric pulp+rice bran (CP+RB), in ratio 1:1, were used in SSF to study their effect on phytase production by *Aspergillus* sp. FS3. The fermentations were carried out in 250-mL Erlenmeyer flasks at 30 °C for 96 h with an initial moisture content of 60 %, pH=5.0 and ammonium citrate as a nitrogen source.

Phytase production in SSF

Fermentation was carried out in 250-mL Erlenmeyer flasks containing CP as the solid substrate and ammonium citrate as a nitrogen source. The moisture level was adjusted to 60 % with ultrapure water. Cotton-plugged flasks were sterilized at 80 °C for 24 h followed by exposure to UV light for 5 h. After cooling down to room temperature, the flasks were inoculated with 10^7 spores/g CP of the corresponding microorganism. The contents of the flasks were thoroughly mixed, then incubated at 30 °C for 96 h, after which the contents were harvested and assayed for phytase activity. All experiments were done in triplicate.

Pretreatment of CP

The effect of different pretreatments of CP on phytase production was studied to reduce microbial contamination (Table 1). The experiments were conducted in 250-mL Erlenmeyer flasks containing CP (Cargill SA, Brazil) as the substrate in SSF. The moisture level was adjusted to 60 % with 0.3 M citrate buffer, pH=5.0. After the pretreatment, the flasks were cooled down to room temperature and inoculated with the soil isolate FS3 or *A. ficuum* NRRL 3135. The contents of the flasks were thoroughly mixed and the flasks were incubated at 30 °C for 96 h. The contents of the flasks were harvested and assayed for phytase activity. All experiments were performed in triplicate.

Table 1. Pretreatment conditions of CP

Pretreatment of CP	Conditions
Control	no thermal treatment, exposure to UV for 2 h
Autoclave (AS1)	dry (moisture 12 %), autoclave (121 °C for 15 min)
Autoclave (AS2)	dry (moisture 12 %), autoclave (121 °C for 15 min)
Autoclave (AU1)	wet (moisture 60 %), autoclave (121 °C for 15 min)
Autoclave (AU2)	wet (moisture 60 %), autoclave (121 °C for 15 min)
Fluent steam (VF20)	wet (moisture 60 %), 100 °C for 20 min
Fluent steam (VF40)	wet (moisture 60 %), 100 °C for 40 min
Fluent steam (VF60)	wet (moisture 60 %), 100 °C for 60 min
Dry incubator (E4)	dry at 65 °C for 4 h, exposure to UV for 2 h
Dry incubator (E8)	dry at 65 °C for 8 h, exposure to UV for 2 h
Dry incubator (E12)	dry at 65 °C for 12 h, exposure to UV for 2 h
Dry incubator (E24)	dry at 65 °C for 24 h, exposure to UV for 2 h
Dry incubator (E48)	dry at 65 °C for 48 h, exposure to UV for 2 h

*AS1, AS2 (CP autoclaved without moisture adjustment); AU1, AU2 (CP adjusted to 60 % of moisture before autoclaving)

Study of different nitrogen sources

After choosing the best substrate for phytase production, the effect of the addition of 0.5 % of different nitrogen sources (ammonium nitrate, ammonium citrate, sodium nitrate, ammonium sulphate, potassium nitrate, ammonium chloride) on phytase production in SSF was studied.

Crude phytase extraction

A mass of 4 g of the fermented mixture was mixed with 40 mL of 0.3 M citrate buffer (pH=5). After maceration, extraction was performed at 4 °C for 15 min while shaking (120 rpm). The extract was clarified by filtration and centrifugation at 4500 rpm for 15 min. The clear extracts were used in a suitable dilution for phytase activity determination.

Phytase assay

Phytase activity was determined by quantification of the phosphate released from phytate during the enzymatic reaction (22). A volume of 1 mL of the clear extract was diluted 1:4 in acetate buffer (pH=5.0) and incubated at 50 °C for 10 min with 1 mL of 1.5 mM phytate dodecasodium salt from corn (Sigma) in citrate buffer, pH=3.0. The reaction was stopped by adding 1 mL of 10 % (by mass per volume) trichloroacetic acid solution (TCA). A blank sample was formed by mixing 1 mL of sodium phytate solution, 1 mL of TCA solution and 1 mL of the diluted extract. Thereafter, 1 mL of distilled water and 5 mL of Taussky-Shorr (TS) reagent as described by Harland and Harland (23) (ferrous sulphate/ammonium molybdate) were added to the blank sample and to the enzymatic reaction. After 10 min at room temperature, absorbance at 660 nm was read. One unit (U) of phytase activity is defined as the amount of enzyme that liberates one μmol of inorganic phosphate per minute under assay conditions.

Inorganic phosphate determination

Spectrophotometric quantification of inorganic phosphate was performed using the Taussky-Shorr reagent. The determinations were performed in triplicate.

Moisture content and water activity

Moisture content was analyzed using a moisture determinator (BEL Engineering Topray BR-001). Water activity (a_w) was obtained by the AQUALAB CX-2.

Results and Discussion

Isolation of phytase-producing microorganisms

Soil, CP, corn, rice bran and wheat bran were used as a source material to screen for phytase-producing microorganisms. The focus of the work was to isolate fungal species producing an extracellular phytase. The main criterion to identify such fungal species was a significant growth on a phytate agar plate. As phytate was the only source of carbon and phosphorus, fungal species capable of growing on such agar plates have to produce an extracellular phytase in order to use phytate carbon and phosphorus. In total, 50 strains showed good growth on phytate agar: 25 strains isolated from soil (FS1 to FS25), 7 from CP pellets (FCP1 to FCP7), 6 from corn (FC1 to FCP6), 8 from rice bran (FRB1 to FRB6) and 4 from wheat bran (FWB1 to FWB4). Phytase production of all strains identified as a phytase producer in this screening was investigated in solid-state fermentation using CP as a substrate. All strains with the exception of 8 also showed significant phytase production in SSF. With 32 U/g, the soil isolate FS3 was the best phytase producer. The reference strain *A. ficuum* NRRL 3135 produced 26 U/g phytase activity under the identical conditions in fermentation. Only 3 further soil isolates showed significant phytase production: FS16 (17.1 U/g), FS22 (10.6 U/g) and FS4 (13.1 U/g), whereas with 6 soil isolates no significant phytase activity was obtained. Strains isolated from CP, corn, rice bran and wheat bran showed relatively high phytase production. Out of 25 isolated strains, 12 produced more than 10 U/g with CP as a solid substrate. The highest phytase production was achieved with FCP2, a CP isolate (12.7 U/g), and FC5, a corn isolate (16.81 U/g).

The course of phytase production of the 12 best phytase producers was studied in SSF at 30 °C using CP as a solid substrate within 24 h (Fig. 1). During the adaptation phase (within the first 24 hours of fermentation), no significant phytase production was observed. Thereafter, all strains began to produce significant phytase activity. Under the conditions used, maximum phytase activity was achieved after about 96 hours of fermentation. Extending the duration of fermentation resulted in a slight decrease in phytase activity, which might be due to proteolytic degradation of the enzyme. The soil isolate FS3, which was shown to be the best phytase producer, was chosen for studies to improve fermentation and enzyme extraction in order to achieve higher yields of phytase activity.

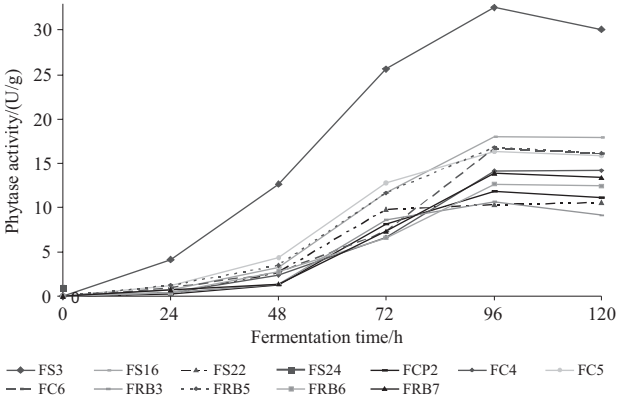

Fig. 1. Course of phytase production during SSF at 30 °C using different fungal isolates

Study of different substrates

The energy required and the physical support for a fungus to grow and to produce the desired metabolite(s) is primarily provided by the substrate (*20*). Thus besides CP, other residues of the agroindustry were studied in respect to phytase production during SSF (Fig. 2). All solid substrates investigated resulted in a significantly

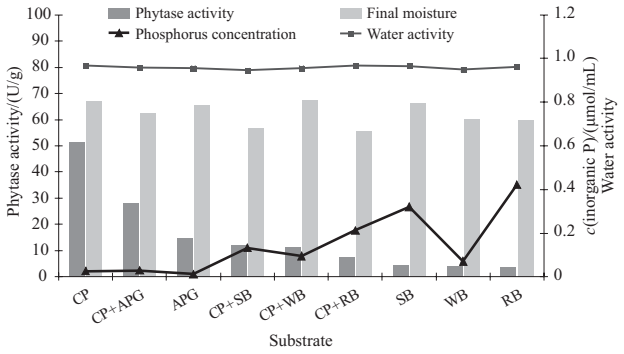

Fig. 2. Phytase production during SSF using different residues of the agroindustry by the soil isolate FS3

lower phytase production compared to CP, although substrates such as SB, WB and RB were commonly used in phytase production by SSF. With WB as a substrate in SSF, phytase activities of about 9 U/g using *Mucor racemosus* NRRL 1994 (*6*) and 1.8 U/g using *A. oryzae* AK9 (*24*) were reported. Using RB as a substrate, phytase activity of even 15.8 U/g was obtained with *A. oryzae* AK9 (*24*). Also, mixtures of the used substrates with CP did not result in an enhancement of the phytase production compared to CP. However, with the soil isolate FS3, the use of WB and RB as substrates resulted in the lowest phytase production. Fig. 2 clearly shows that phytase production during SSF is well correlated with the initial concentration of inorganic phosphate in the substrates used. Phosphate is a well known repressor of phytase synthesis in many fungi (*21*). Vohra and Satyanarayana (*25*) reported that maximum phytase production occurred in a medium containing only 5 μg/mL of inorganic phosphate, and higher concentrations resulted in a repression of phytase synthesis. This is in good agreement with the results reported here. Phytase production was better in substrates with a low concentration of inorganic phosphate such as CP (0.025 μmol/g of inorganic P), APG (0.028 μmol/g of inorganic P) and CP+APG (0.011 μmol/g of inorganic P), whereas SB, RB and WB contained higher concentrations of inorganic phosphate.

Study of nitrogen source

As indicated in Fig. 3, the nitrogen source used during SSF could greatly affect phytase production. Supplementation with a nitrogen source resulted in a better phytase production compared to the control without supplementation. Maximum phytase production was observed with both the control strain *A. ficuum* and the soil isolate FS3 when ammonium nitrate was used as a nitrogen source. This is in agreement with previous studies using *A. niger* van Tieghem as the fermentation organism (*21*). Supplementation with ammonium citrate and ammonium sulphate resulted also in a significant increase in phytase production compared to the unsupplemented controls.

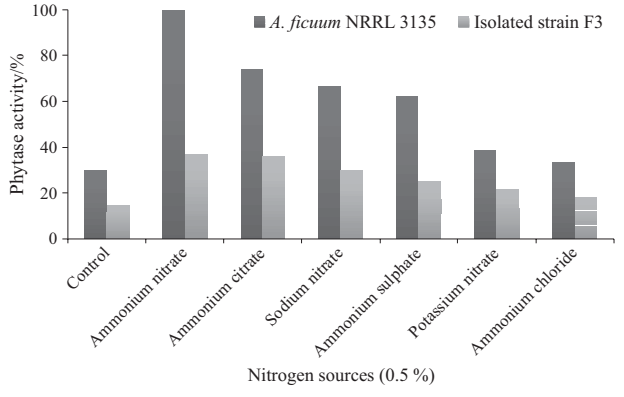

Fig. 3. Effect of different nitrogen sources on phytase production by *A. ficuum* NRRL 3135 and soil isolate FS3 during SSF using CP as a solid substrate

Effect of CP pretreatment on phytase production

Fig. 4 clearly shows that pretreatment of CP affects phytase production by the soil isolate FS3 during SSF. The best phytase production (44.83 U/g) was observed after treating CP in a dry incubator for 4 h at 65 °C (E4). Longer drying times, higher drying temperatures, higher moisture content as well as autoclaving resulted in a significant lower phytase production during SSF. It could be suggested that these pretreatments resulted in a release of inorganic phosphate from phosphorylated compounds present in CP.

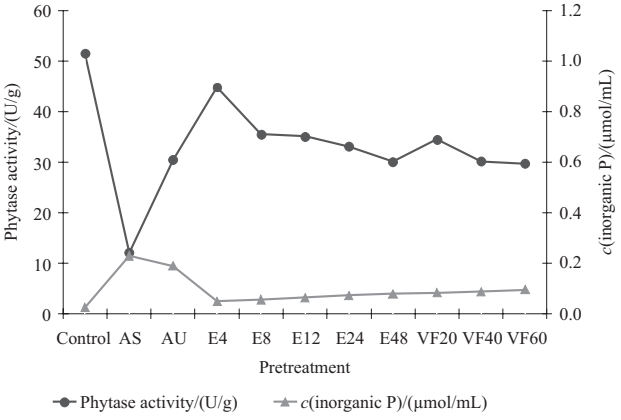

Fig. 4. Effect of CP pretreatment on phytase production during SSF by FS3

Conclusions

Although a great number of fungi producing extracellular phytase could be isolated from natural habitats, only 12 strains (soil isolates: FS3 (32.2), FS16 (17.1), FS22 (10.6), FS24 (13.1), CP isolate: FCP2 (12.7), corn isolates: FC4 (14.7), FC5 (16.7), FC6 (16.8), rice bran isolates: FRB3 (10.9), FRB5 (16.9), FRB6 (12.5), (FRB7 13.9) achieved phytase activities of above 10 U/g in SSF using CP as a substrate. The soil isolate FS3 was identified as the best phytase producer. This strain was even slightly better (32 U/g) than the reference strain *A. ficuum* NRRL 3135 (26 U/g). Although the substrates PG, SB, WB, RB also promote phytase synthesis by *A. ficuum* NRRL 3135, the yields were not as high as that obtained from CP. Combination of CP and the other substrates resulted in lower phytase production compared to CP itself. Thermal pretreatment of CP also resulted in a decreased phytase production. However, exposure to UV and a high inoculum rate have been proven sufficient to prevent growth of microbial contaminations during SSF.

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