Optimized production of phytase by solid-state fermentation using triticale as substrate and source of inducer

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This study was carried out to evaluate the process of phytase production by Aspergillus niger in solid-state fermentation (SSF) using triticale waste. A waste that currently has no use was reported for this biotechnological process, and is of high impact due to the null use. The process was carried out using an additive free medium, supplemented with only one nitrogen source. Under these conditions, phytase activity of 7.45 U/g dry substrate (DS) was obtained. The process was optimized using different additives such as dextrose, lactose, Tween 80 and potassium chloride. For fermentation maximization, two experimental designs were used: 1) Plackett-Burman design (PBD) and 2) the Box-Behnken design (BBD). PBD was used to evaluate the effect of related variables on the production of phytase, as well as their level of significance in the process, while BBD was used for optimal conditions determination. The process was conducted with Petri dishes and a maximum enzyme activity of 25.8 IU/g DS was obtained. Subsequently, SSF was carried out in a tray to increase the amount of fermented substrate and phytase activity of 23.63 IU/g DS was obtained. The results of this study suggest a minimal decrease (8.4%) in enzyme production with scaling.

Key words: Triticale, solid-state fermentation, phytase, Aspergillus niger, optimization, statistical experimental design.

INTRODUCTION

Solid state fermentation (SSF) is a technique which has been known for hundreds of years. It is defined as a metabolic process by which organisms grow on a solid matrix, in the absence or near absence of free water (Díaz et al., 2007; Pal and Khanum, 2010). Substrates used in SSF process are generally not soluble in water, and are integrated as carbon source, vitamins and minerals; therefore, they must have enough moisture to
support the growth and metabolic activities of microorganisms (Ali and Zulkali, 2011; Graminha et al., 2008). In recent times, SSF has been used with great success in the production of a large number of metabolites of interest, including antibiotics, surfactants, organic acids, aromatic compounds, pesticides and many enzymes. In this process, the incorporation of agroindustrial wastes mainly as substrates which are beneficial, is needed for the production of metabolites of interest, and is accessible at no cost (Bhavsar et al., 2010; Diaz et al., 2007; Haefner et al., 2005; Pandey et al., 2001).

Phytases are hydrolytic enzymes, phosphatases type, and belong to the subfamily of histidine acid phosphatases. They are responsible for catalyzing the hydrolysis of phytate phosphor mono ester bonds (salts myo-inositol hexakisphosphate) or myo-inositol 1,2,3,4,5,6-hexakisdihidrogeno phosphate (phytic acid) producing derivatives, such as tetra, tri, di and inositol monophosphate and inorganic phosphate (Pi) (Shivanna and Venkateswaran, 2014; Albarracín et al., 2013; Bilgiçli et al., 2006). This enzyme is mainly applied in the animal feed industry, where it is used as supplement in feeds of non-ruminant animals (such as pigs, chickens, turkeys, etc.). This is because phytic acid is the largest reservoir of phosphorus in plant, with 60 to 80% bound to different compounds. In monogastric animals, phosphorus is largely unavailable in phytic acid due to the absence of phytase in their digestive system. This leads to the elimination of phytic acid via stool, which consequently results in soil pollution and eutrophication of water by phosphates (Fei et al., 2013; Ma et al., 2011; Vats et al., 2009).

Phytase is an enzyme that liberates the Pi present in phytic acid and makes it available for digestion in animals (Vats et al., 2009; Velayudhan et al., 2015). When used as a supplement, it has been shown to reduce the Pi in manure by about 33%, which ensures a third less environmental pollution and improvement of animal nutrition. The main limitation to the use of this enzyme with high nutritional and environmental interest is the high market price and, in some cases, lower levels of production (Haefner et al., 2005; Romero et al., 2009).

This enzyme is produced by submerged fermentation (SF) or by solid substrate fermentation (SSF). Studies have shown that the best alternative method used for the production of this enzyme is through the application of SSF (Haefner et al., 2005; Marlida et al., 2010; te-Biesebeke et al., 2002). Phytase can be directly produced in SSF by filamentous fungi using some agroindustrial residues as substrate to add value to the process, and supplemented with various additives to favor its production (Pal and Khanum, 2010). The rigorous processes of SSF were recently described to optimize the production process and, at the same time, to maximize yield (Bhavsar et al., 2010; Saad et al., 2011). The classification of this enzyme as Generally Recognized as Safe (GRAS), provides a large field of study because of the great benefits it brings to phytase, and the relative ease with which it is produced. This is intended to intensify both the search for new microorganisms, as well as abundant waste utilization, with little or no market value. This provides an environment that is similar to the native environment of microorganism for the development and production of adequate metabolites.

Triticale (Triticosecale Wittmack) is a synthetic crop species developed by crossing wheat and rye. Its peculiar name originates from the union of the scientific names of the two genera involved: wheat (Triticum) and rye (Secale). The peculiarity of triticale is that it combines the features of rye genome, such as hardiness, disease and environmental tolerance, with the high yield potential and grain quality of wheat (Cantale et al., 2016). These features make it a suitable crop for any environment with little or no susceptibility to biotic stress. Thus, this saves the cost of production as compared to other crops. This resulted in high grain yield, low loss and high biomass production. Besides its great culture qualities, it has been found to be beneficial in the recovery of contaminated soil, and in promoting the growth of carbon-fixing microorganisms (Borneo et al., 2016; Giunta et al., 2015). Therefore, despite being a new crop, it has been shown to have huge benefits as compared to other crops. The growing interest in its production can be attributed to the increasing amount of soil intended for cultivation, resulting in an increase in unused materials, thereby causing waste (Jondreville et al., 2007). This provides a great opportunity for the incorporation of these residues in the biotechnological production processes of industrial metabolites of high interest. Thus, one of the main objectives of this study was to evaluate a sufficiently produced waste in the state of Coahuila, Mexico, which has been previously studied as livestock feed. Therefore, the objective of this study was to use triticale agroindustrial waste as a substrate for the production of phytase by fermentation in solid medium and maximize production levels by improving the nutritional parameters of the medium.

**MATERIALS AND METHODS**

**Fungus and inoculum preparation**

The Aspergillus niger 7A-1 strain was provided by the
Nanobioscience Group, University Autonoma of Coahuila, Saltillo, México. It belongs to a group of strains isolated from the semi-desert of Coahuila, México. Prior to the commencement of the study, the strain was grown and maintained on potato dextrose agar (PDA) slants at 28 ± 1°C, to obtain the inoculum. After seven days of fungal growth, spores were collected with 0.1% Tween 80. Their concentration was adjusted to 1 × 10^8 spores/mL (Bhavsar et al., 2010).

**Designs of SSF culture medium**

Substrates used for the production of phytase enzyme were obtained from the experimental fields of the Agrarian Autonomy University, “Antonio Narro”. The substrate was washed with distilled water to remove soil and impurities and dried at 60°C. It was separated in spike and stems, then ground to obtain a particle size of approximately 0.3 mm and stored separately in sealed bags until use. Three different mixtures were made between spike and stems of the residues, which is defined in Table 1.

**SSF in Petri dish**

Five grams (5 g) of dry substrate (DS) was placed in a Petri dish and sterilized at 121°C for 20 min. After cooling, the substrate was supplemented with 3 mL solution containing NH_4NO_3 4% (w/v), previously sterilized. The substrate was inoculated with 0.5 mL spore suspension of fungus at 1 × 10^8 spores/mL (Bhavsar et al., 2010). The Petri dish was incubated for 5 days at 28 ± 1°C. The moisture content of the inoculated substrate was approximately 60%. All the experiments were carried out in triplicate and the average values were taken.

**SSF scaling tray**

500 g of selected blend previously dried, was placed in an aluminum tray (300 mm x 150 mm x 60 mm) with a layer thickness of 3 cm. Thereafter, it was inoculated with 50 mL of spore solution, and the moisture was adjusted to 60% with the mentioned optimized medium. The trays were covered with plastic wrap, incubated at 28 ± 1°C for 5 days with 99% relative humidity in a chamber. They were ventilated twice every day for five minutes. The trays were sampled every 24 h under aseptic conditions to obtain a representative sample of 2 g. The substrate and the solution (with adjusted moisture) were sterilized at 121°C for 20 min before use.

**Enzyme activity assay**

The phytase activity was determined in crude extract (SSF) obtained from sample with distilled water (5 mL/g sample) and stirred for 1 h (200 rpm at 25 ± 1°C). The suspension was centrifuged (10,000 g for 10 min) and the supernatant (crude extract) was stored at 4°C until further utilization (Mittal et al., 2011).

Phytase activity was determined by measuring the Pi released from sodium phytate solution (Harland and Hraland, 1980; Monteiro et al., 2015). The reaction mixture consisted of 1 mL of 0.1 M MgSO_4·7H_2O, 2.4 mL of 6.82 mM phytic acid and 0.6 mL of appropriately diluted crude enzyme solution. Solutions of MgSO_4·7H_2O and phytic acid were prepared with 0.2 M sodium acetate buffer (pH 5.15). Subsequently, the reactants were incubated at 55°C for 60 min, and the reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid. Thereafter, 1 mL of distilled water and 2.4 mL of Taussky-Schorr reagent (10 mL of 10 N H_2SO_4, 1 g of (NH_4)MoO_4·4H_2O and 5 g of FeSO_4·7H_2O graduated to 100 mL distilled water) were added to generate a blue chromophore (Harland and Hraland, 1980). The content was mixed for 30 min and then the absorbance was determined at 660 nm. Measured values were correlated with a standard curve that was constructed using monopotassium phosphate. One unit of phytase activity was defined as the amount of enzyme that released 1 µmol of phosphate per minute under assay conditions. All the enzyme activity analyses were performed in triplicate.

**Other analytical determinations**

Protein concentration was determined by the Bradford method using bovine serum albumin as the standard at 0 to 20 µg/mL (Bradford, 1976).

**Experimental design to optimize the medium selected and statistical analysis**

The aim of applying this experimental design was to identify components that promote the high production of phytase enzyme. The Plackett-Burman (PBD) experimental design facilitates the analysis of multiple independent variables in a process, and help in demonstrating their significance (Liu and Tang, 2010). Several studies have identified seven variables that are effective in the production process (Bhavsar et al., 2010; Mittal et al., 2011). Two reference values for each variable were used, the higher level was denoted by ‘+’ and the lower by ‘−’ (Table 2).

The experiments were carried out in Petri dishes with 5 g of medium selected, to which was added a medium constituting seven additives, two carbon sources, one surfactant, three mineral sources and one phosphoric acid for pH adjustment (Table 2) under the conditions described above at constant 60% moisture content. The SSF was carried out under the conditions described above. The effect level of each selected variables was determined by the difference between the average responses. The significance level of each variable was determined by Pareto charts, and statistical analyses of the data were carried out using analysis of variance (ANOVA) and Student’s t-test mean comparison test (p<0.05) (Statistica® 7, Statsoft, 217 Tulsa, OK, USA). The Box-Behnken design (BBD) was used to investigate the relationship between variables of medium components, and to optimize their concentrations for the best yield of phytase enzyme production in SSF, using A. niger (Bogar et al., 2003; Mittal et al., 2011). In experiments of the present study, four factors at three levels of variations were defined (Table 5). Subsequently, results were obtained through the application of analysis of variance (ANOVA). To design the experiments, analysis of variance and process maximization were done using Statistical Package 7 for Windows, for regression analysis of the experimental data obtained. To determine the significance of regression coefficients, a t-test was applied. All experimental designs were randomized. The experiments were carried out in triplicate in Petri dishes, and the mean values were recorded.

**Table 1. Mixtures used for the preselection of SSF medium for the production of phytases by A. niger 7A-1, based on their content of spike and straw.**

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Spike (%)</th>
<th>Straw (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>M2</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>M3</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Selection of the mixture appropriate for phytase production

The mixtures analyzed for the production of phytase enzyme yielded highly significant results. In the M3, the amount of enzyme produced was the lowest. 3.67 IU/g was probably due to the high concentration of nutrients contributed by spike, thus avoiding the need for the microorganism to synthesize the enzyme of interest. Also, concentrations of spike and forage in equal parts, (M2) only produced 5.76 IU/g DS, which can be attributed to the fact that the fermenting medium has a larger compaction and this makes the aeration processes difficult. For this reason, M1 was selected for obesity studies. With this composition, it was possible to obtain 7.45 IU/g DS of phytase by having a concentration of nutrients that allows the induction of phytase and maintains an adequate aeration atmosphere allowing the proper development of the microorganism.

Screening of significant nutrients using the Plackett-Burman design (PBD) for SSF by A. niger

In the basal medium selected, 7.45 IU/g DS was produced in the fifth day of fermentation (Figure 1). The PBD for eight trials with two levels of concentrations were carried out to evaluate the significance of the seven medium components shown in Table 3 (Zhang et al., 2006). This table also shows the phytase activity obtained in each of the experiments, wherein an extensive difference between each of the treatments was observed. Between 10 and 40%, an increase in the levels of phytase activity (Table 3) was obtained. This variation demonstrates the importance of medium supplementation in achieving higher productivity. These results are similar to those reported by Bhavsar et al. (2010) where A. niger managed to raise phytase enzyme production by 34% using waste wheat and various additives.

To differentiate the important process variables in SSF and their degree of influence, Pareto chart was used (Figure 1). The chart shows bars with a length proportional to the value of the effect on the studied process. The bars are displayed in accordance with the size of effects, with the largest effects on top. The diagram for any individual effect allows an evaluation of the probability of finding the observed random effect (Meena et al., 2013). Figure 1 shows a vertical line at the critical t-value α of 0.50, and the effect for which the bar is smaller than the critical t-value is considered variable, having no significant effect on the studied process.

Table 4 shows the total sum of squares and respective percentage contribution for each variable. The results are also shown in Figure 1. It is also shown that the variable E, F and G, have a negligible contribution. D (KCl) showed greater significance in process, similar to that reported by Bhavsar et al. (2010) who used A. niger and wheat based medium supplemented with KCl among others, to show the importance of this compound using PBD.

The highest phytase activity obtained was 12.5 IU/g DS. This result was obtained in the basal production medium containing only the solid substrate and 40 g/kg (NH₄)₂NO₃ as source of nitrogen after 120 h of incubation. It was found to be 40% higher than the initial 7.45 IU/g DS.

The coefficient of regression obtained was $R^2 = 0.9314$ and it was shown that the model used for analyzing the data is significant. This indicates that the model explains 93% of the variability in data. Four medium components (Table 3) were identified as significant like variables for phytase production by BBD and after model optimization, BBD was used. According to Singh and Satyanarayana (2011), carbon source plays an important role in the production of enzymes, in particular, the use of simple sugars as an important variable for phytase production. Besides that, it directly influences the regulation of moisture levels by interacting with solutes present in the SSF medium (Mittal et al., 2011).

Table 5 shows the value used in maximization. Table 6...
Figure 1. Pareto bar charts for the estimation of effects (absolute values) of the independent variables (factors) present in the SSF for phytase production by A. niger 7A-1. The extent of the bars along the vertical dotted line ($p = 0.05$) represents the significance dimension.

Table 3. Variables analyzed in the PBD design and its response as a function of phytase activity yields detected for each variable for improvement in yields of phytase enzyme produced by A. niger 7A-1.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Variables</th>
<th>Phytase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
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<td>5</td>
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<td>6</td>
<td>1</td>
<td>-1</td>
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<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4. Percent of contribution and ANOVA of each variable and its effect on phytase production in triticale waste by A. niger 7A-1.

<table>
<thead>
<tr>
<th>Variable code</th>
<th>Sum of squares</th>
<th>Contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>17.85</td>
<td>1.78</td>
</tr>
<tr>
<td>B*</td>
<td>16.03</td>
<td>1.60</td>
</tr>
<tr>
<td>C*</td>
<td>15.58</td>
<td>1.55</td>
</tr>
<tr>
<td>D*</td>
<td>21.54</td>
<td>2.15</td>
</tr>
<tr>
<td>E</td>
<td>1.47</td>
<td>0.014</td>
</tr>
<tr>
<td>F</td>
<td>0.31</td>
<td>0.003</td>
</tr>
<tr>
<td>G</td>
<td>3.66</td>
<td>0.036</td>
</tr>
</tbody>
</table>

*Highly significant.
Table 5. Levels used for the BBD design of each variable that showed significant difference on phytase production by *A. niger* 7A-1 in SSF.

<table>
<thead>
<tr>
<th>Code</th>
<th>Variables</th>
<th>Levels</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1</td>
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<tr>
<td>A</td>
<td>Dextrose %(w/w)</td>
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</tr>
<tr>
<td>B</td>
<td>Lactose %(w/w)</td>
<td>0.2</td>
</tr>
<tr>
<td>C</td>
<td>Tween 80 %(v/w)</td>
<td>1.0</td>
</tr>
<tr>
<td>D</td>
<td>KCl mg/g DS</td>
<td>0.15</td>
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Table 6. BBD matrix and result of experiments by phytase production with *A. niger* 7A-1 using triticale waste.

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Factor level</th>
<th>Response of phytase activity (IU/g DS)</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
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<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>-1</td>
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<td>26</td>
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</tr>
<tr>
<td>27</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

shows the design matrix of four significant variables in coded levels and reported experimentally, the phytase activity obtained. The trial number 27 had the highest phytase activity with 17.41 IU/g DS. The experimental results obtained for phytase production were analyzed using backward elimination regression. The regression coefficients, t- and P-values, are present in Table 7. Following the analysis, the quality of the obtained model was evaluated on various criteria. One is their correlation coefficient $R^2$, which was 0.9561 for phytase production. In terms of percentage, this indicates that 95.61% corresponds to variability in the aforementioned model. The value of the correlation coefficient predicted for phytase production was 0.9383, which shows a strong agreement between the experimental and predicted values of phytase production. The coordinates of the maximum point found are: $\text{A}=1.2$, $\text{B}=1.2$, $\text{C}=1.2$ and $\text{D}=-0.2$, corresponding to the optimal supplementation levels of dextrose with 16.8 g, lactose 0.48 g, Tween 80 1 mL and KCl 0.2 g for each 100 g of triticale. In further experiments, triticale was supplemented with these optimized levels of additives. The results obtained in the present study showed a strong correlation agreement between predicted and experimental response.

Phytase production under maximized fermentation conditions was studied for 144 h. Figures 2 and 3 shows the observed difference between the optimized and un-
Table 7. Results of regression analysis of BBD in phytase production by *A. niger* 7A-1 in SSF with triticale waste. Coefficient of determination, $R^2=0.95$.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>$t$-value</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>13.0368</td>
<td>50.7015</td>
<td>0.000000</td>
</tr>
<tr>
<td>A</td>
<td>0.9754</td>
<td>5.0896</td>
<td>0.000005</td>
</tr>
<tr>
<td>B</td>
<td>1.0667</td>
<td>5.0808</td>
<td>0.000005</td>
</tr>
<tr>
<td>C</td>
<td>1.0412</td>
<td>6.0474</td>
<td>0.000000</td>
</tr>
<tr>
<td>D</td>
<td>-0.1215</td>
<td>-1.0028</td>
<td>0.320390</td>
</tr>
<tr>
<td>AB</td>
<td>0.2497</td>
<td>0.2746</td>
<td>0.784628</td>
</tr>
<tr>
<td>AC</td>
<td>-0.0663</td>
<td>-0.7297</td>
<td>0.468679</td>
</tr>
<tr>
<td>AD</td>
<td>0.3112</td>
<td>3.4333</td>
<td>0.001188</td>
</tr>
<tr>
<td>BC</td>
<td>-0.1040</td>
<td>-0.9914</td>
<td>0.325891</td>
</tr>
<tr>
<td>BD</td>
<td>-0.4980</td>
<td>-0.4750</td>
<td>0.636655</td>
</tr>
<tr>
<td>CD</td>
<td>-0.2271</td>
<td>-2.1640</td>
<td>0.034900</td>
</tr>
<tr>
<td>$A^2$</td>
<td>0.1219</td>
<td>0.3673</td>
<td>0.714767</td>
</tr>
<tr>
<td>$B^2$</td>
<td>1.0565</td>
<td>5.0325</td>
<td>0.000000</td>
</tr>
<tr>
<td>$C^2$</td>
<td>0.2810</td>
<td>1.3387</td>
<td>0.186271</td>
</tr>
<tr>
<td>$D^2$</td>
<td>-0.5950</td>
<td>-2.8343</td>
<td>0.006447</td>
</tr>
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</table>

Table 8. Comparison of phytase production by other fungal strains under SSF.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Production Technique</th>
<th>Substrate</th>
<th>Phytase activity (IU/g DS)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus ficuum</em></td>
<td>SSF</td>
<td>Wheat bran</td>
<td>15</td>
<td>Bogar et al. (2003)</td>
</tr>
<tr>
<td><em>Aspergillus niveus</em></td>
<td>SSF</td>
<td>Wheat bran</td>
<td>3.4</td>
<td>El Gindy et al. (2009)</td>
</tr>
<tr>
<td><em>Mucor Racemosus</em></td>
<td>SSF</td>
<td>Groundnut oil cake</td>
<td>24.3</td>
<td>Roopesh et al. (2006)</td>
</tr>
<tr>
<td><em>Rhizopus oligosporus</em></td>
<td>SSF</td>
<td>Coconut oil cake</td>
<td>30.1</td>
<td>Ramachandran et al. (2005)</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>SSF</td>
<td>Coconut oil cake</td>
<td>27.6</td>
<td>Ramachandran et al. (2005)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>SSF</td>
<td>Triticale wasted</td>
<td>25.8</td>
<td>Present work</td>
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</tbody>
</table>

Optimized models. The maximized medium showed a phytase production of 7.45 IU/g DS, whereas maximization studies gave phytase production of 25.80 IU/g DS on the 5th day of fermentation. These results showed that phytase activity increased by 3.4 times in the maximized medium experiment.

The maximized results showed a productivity of 4,300 IU/kg/day. The results of productivity obtained in this study are highly competitive as compared to other previous studies. For example, Krishna and Nokes (2001) obtained a productivity of 4,667 IU/kg/day with *A. niger*. In their study, it was shown that fungi of the genus *Aspergillus* are the best producers of phytase (Bhavsar et al., 2013; Soni et al., 2010). Table 7 shows the results of other researches on the production of phytase with various agroindustrial residues. These data confirmed the competitiveness of the microorganism used in the present investigation against other microorganisms used for the production of phytase. Also, the data highlight the importance of triticale waste used in this research, because, the use of this waste in the production of enzymes is yet to be reported. The results showed that this residue is of value as compared to other highly studied agro-industrial residues, such as wheat bran or canola cake, in the production of phytase and other hydrolases (Table 8).

**Estimation of phytase production by SSF**

Given these results and considering that the requirements of phytase are 400 IU/kg of food. The crude extract of 15.5 kg of triticale was fermented to treat a metric ton (MT) of feed. An equivalent amount is needed to maintain 10 pigs per month (considering that a pig consumes between 3 and 4 pounds of feed per day). Definitely, it is highly profitable, taking into account that the cost of fermentation of substrate needed to treat a metric ton is US$ 28.48. Finding products to market reaches US$ 300 with only half prepared for deal in metric ton.

To confirm these results, an encouraging image was presented indicating the pilot production level. Products rich in phytase enzyme also contain some other hydrolytic enzymes that favor digestion in non-ruminants. This was reported by Bogar et al. (2003) who found alpha amylase, xylanase and others. The supplementation of these enzymes together with phytase contributes to a
Figure 2. Kinetics of phytase production in SSF by *A. niger* 7A-1 under optimized conditions (filled squares) and without optimization (filled triangles).

Figure 3. Kinetics of 6 days SSF by *A. niger* 7A-1 in a tray. Fermentation conditions: 500 g triticale dried on a tray (300 × 150 × 60 mm) under the conditions of optimization and initial moisture of 60% and 28±1°C.

decrease in the viscosity of food during digestion, amount of loose droppings, increases or mass gain, feed conversion and egg production in the case of poultry (Morales et al., 2011; Romero et al., 2009).

**Up-scaling production of phytase enzyme by SSF in trays**

SSF was performed in stainless steel stationary trays.
The procedure was scaled-up from 5 g waste triticale in Petri dish to 500 g waste triticale in trays: 300 mm × 150 mm × 60 mm. By scaling up from Petri dishes to stationary trays, the activities of 25.8 IU/g DS were reproducibly obtained. These results are therefore encouraging for maximization under pilot scale conditions. The scale up showed an 8.4% reduction in activity obtained in the Petri dish, which can be attributed to the enlargement process in the model.

Conclusion

Based on analysis of the results of this research, it can be concluded that the use of triticale as waste for the production of phytase, is highly feasible both from an economic point of view, as well as performance. The results obtained are highly significant and can lead to further investigation due to the importance of phytase in industrial animal feed. Both A. niger 7A-1 and triticale residue used showed high efficiency in the process of maximizing the production of phytase with minimum nutritional requirements. Keeping highly affordable process and increasing production is of great interest at the industrial production level. It should be noted that the total SSF process is a sustainable process which demonstrates the importance of using the null value of this residue in the production of metabolites. The test level scale showed a high feasibility of applying this waste for industrial phytase enzyme production while maintaining high yields, efficiency and low processing requirements cost. However, the results obtained in this study are of great importance, since the use of triticale as waste in the production of phytase showed high competitiveness against waste used industrially for commercial phytase production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Morales GA, Moyano FJ, Marquez L (2011). In vitro assessment of the


