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Article

Optimization of Phytase Production from *Escherichia coli* by Altering Solid-State Fermentation Conditions

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Abstract: Cultivation of *Escherichia coli* on wheat-bran substrate under various Solid-State Fermentation (SSF) conditions was evaluated for phytase yield along with the enzyme activity profile as a potential, low-cost alternative to submerged-liquid fermentation. The maximum phytase activity achieved by *E. coli* was 350 ± 50 SPU of phytase activity per gram of bran, incubated for 96 h with a substrate bed moisture content of 70% (w/v) at 37 °C with a relative air humidity of 90%, and supplemented with 10% (w/w bran) Luria-Bertani broth powder which translates into a 300% increase in phytase activity compared with an un-supplemented culture. The greatest improvements in phytase yield were associated with nutrient supplementation and the optimization of initial substrate moisture content. *E. coli* production of phytase utilizing solid-state fermentation technology was shown to be feasible utilizing the low-cost agro-residue wheat bran as substrate. Furthermore, the effect of pH and temperature on phytase activity was monitored from pH 2.5 to pH 7.5, and for temperatures ranging from 20 °C to 70 °C. Optimal phytase activity was at pH 5.5 and 50 °C when produced under the SSF optimized conditions.

Keywords: *E. coli;* enzyme; optimization; phytase; solid state fermentation

1. Introduction

Monogastric livestock lack the enzyme phytase needed to digest phytate, the predominant form of the essential nutrient phosphorus (P) in grains. To make more efficient use of the phosphorus in feed, diets are routinely supplemented with exogenous phytase [1]. Phytase supplements used in animal nutrition are typically of microbial origin, with commercial enzyme production by means of solid-state fermentation (SSF) or submerged liquid fermentation (SLF). Solid-state fermentation is both economically and environmentally advantageous in that SSF cultivation can be carried out in simpler and therefore more cost-effective bioreactors; the enzymes produced typically can be used directly in their crude form without need for purification or concentration steps [2], which negates the need for capital and energy input; there is a significant reduction in effluent disposal and/or treatment cost, because there is no need to remove vast amounts of water from the product steam [2], and low-cost, nutrient-rich agro-residues can be recycled as substrates for enzyme cultivation [3]. Currently, the majority of commercial SSF phytase is produced by growing the fungus *Aspergillus niger* on wheat bran, which provides both a surface area for microbial attachment and carbon and nitrogen nutrients from xylan and protein [4].

However, bacterial phytases offer some distinct advantages in terms of their stability and resistance to proteolysis over phytases synthesized by fungi [5]. Traditionally, because of moisture requirements, the commercial production of bacterial enzymes has been achieved by SLF, which utilizes free-flowing substrates (e.g., molasses, broth). SSF technology offers many technical and economic advantages over SLF, which is why the commercial potential of bacterial phytase production using SSF technology has been of increased interest. Indeed, research has shown that SSF production of phytase by *Bacillus* spp. is economically feasible when process conditions are optimized to enhance enzyme yields utilizing low-cost substrates [6]. In contrast, whilst studies confirm that *Escherichia coli* can express phytase that is stable under high temperatures and resistant to proteolysis [7], very little information has been published that details *E. coli* phytase production under SSF conditions. To address this knowledge gap we evaluated the effects of solid-state fermentation process conditions on phytase yield from *E. coli* cultivated on a wheat bran substrate.

2. Experimental Section

2.1. E. coli Inoculum

Luria-Bertani (LB) broth containing 25 g dehydrated LB (Difco, Sparks, MD, USA) per liter was autoclaved at 121 °C for 15 min, after which a 1-mL aliquot of *E. coli* (pAPPA1 plasmid in *E. coli* (ATCC 87441™)) stock culture (stored at −80 °C) was added. The prepared culture was transferred to a shaking incubator (37 °C, 200 rpm) and typically grown for 8 h until it attained approximately 3.15×10^7 CFU mL^{-1}.

2.2. Solid-State Fermentation

After completion of the liquid cycle, the culture was transferred to a wheat bran substrate to initiate the solid-state fermentation. Five grams of soft, coarse wheat bran (Siemer Milling, Hopkinsville, KY, USA)

was sterilized in an autoclave at 121 °C, 15 PSI for 20 min in a 125 mL wide-necked Erlenmeyer flask covered with a Bio-Shield wrap (Figure 1). The depth of the bran-bed was 1.3 cm inside the flask, which corresponded to a surface area to volume ratio of 0.76, allowing for sufficient air exchange during growth. The pre-grown liquid culture was mixed with sufficient sterile deionized water to achieve an inoculum of approximately 1.13×10^7 CFU g^{-1} at 60% (*w/v*) SSF bed moisture content. The inoculated flasks were placed into a Forma Scientific Incubator (Model 3033, Marietta, OH, USA) at 37 °C and 90% humidity. The cultures were incubated without agitation. Phytase activity was measured after SSF completion at predetermined times in response to varied process conditions: nutrient additives, substrate moisture level, inoculation rate, and incubation period. Enzyme activity of the phytase was evaluated by creating a temperature and pH profile. All experiments were completed in triplicate.

Figure 1. Erlenmeyer flask containing 5 grams of sterilized inoculated wheat bran.

2.3. Effect of Nutrient Additives on Phytase Production

The following nitrogen-rich nutrient additives were individually tested: yeast extract, LB powder, and tryptone. (Both yeast extract and tryptone are components of LB powder.) Nutrients were added at concentrations of 10, 50, 100, and 250 $mg \cdot g^{-1}$ bran. Phytase production was measured for each nutrient concentration utilizing 1.13×10^7 CFU *E. coli* g^{-1} of wheat for 96 h at 37 °C.

2.4. Effect of Substrate Moisture on Phytase Production

The influence of moisture on enzyme production was evaluated by varying the amount of water applied to bran in addition to the standard inoculum. Moisture levels of 40, 50, 60, 70, and 80% (*w/v*) were established, as determined by a Mettler Halogen Moisture Analyzer (Model HR83, Columbus, OH, USA). Water activity was determined using a water activity meter (Model CX-2, AquaLab, Pullman,

Washington, USA). Moisture levels were maintained by keeping the humidity inside the incubator at 90%. Phytase production was measured for each moisture level utilizing 1.13×10^7 CFU *E. coli* g^{-1} of wheat bran for 96 h at 37 °C.

2.5. Effect of Inoculation Rate on Phytase Production

Culture flasks containing 5 g of sterile bran were inoculated with an 8 h bacterial culture. The overall moisture level of the substrate was maintained at 60%, while five inoculum rates were established: 4.54×10^7, 2.27×10^7, 1.13×10^7, 5.4×10^6 and 9.07×10^5 CFU g^{-1} bran, representing culture to water ratios of 1:1, 1:2, 1:4, 1:10 and 1:50, respectively. Phytase activity in response to each inoculum rate was measured after 96 h of incubation at 37 °C.

2.6. Effect of Incubation Period on Phytase Production

Flasks were prepared containing *E. coli* at approximately 1.13×10^7 CFU g^{-1} of bran. Substrate moisture was maintained at 60% (w/v) at 37 °C. Phytase production was measured after 24, 48, 72, 96, 120, 144 and 168 h of incubation.

2.7. Effect of Temperature and pH on Phytase Activity

Two-gram samples of dried SSF substrate were assayed for phytase activity at 20, 30, 40, 50, 60 and 70 °C at each of six pH levels: 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5. The SSF substrate used had been grown under the following conditions: 60% (w/v) moisture, 1.13×10^7 CFU *E. coli* g^{-1} of bran, 10% LB broth powder, for 96 h at 37 °C.

2.8. Phytase Activity Assay

Incubation was stopped by adding an ammonium molybdate/acetone reagent, which produces a colored complex. Phytase production was determined by assaying phytase activity based on the amount of ortho-phosphate released by enzymatic hydrolysis of sodium phytate under controlled conditions detailed in Engelen *et al.* (1994). The color absorbance of the ortho-phosphate was measured at 380 nm. One solid-state fermentation phytase unit (SPU) is defined as the amount of enzyme required to liberate 1 μmol of inorganic phosphate per minute at pH 5.5 and 50 °C. A control blank containing stop solution was run simultaneously against test solutions. All other reagents were added and read at 380 nm against a water blank. The blank absorbance was subtracted from the sample absorbance and the standard curve. All measurements were performed in triplicate and the respective means reported.

2.9. Statistical Analyses

One-way analysis of variance (ANOVA) was performed to compare the differences between means; regression analyses were performed to identify effects of independent variables on enzyme production. Significance was declared at $p < 0.05$. All analyses were performed utilizing Minitab software (State College, PA, USA).

3. Results and Discussion

3.1. Effect of Additives on Phytase Production

Because the effect of nitrogen supplementation varies between nitrogen sources and organism species, testing to identify optimal rates is useful [8]. Wheat bran substrate typically offers an abundant source of carbon to support microbial growth; however, supplementation of other growth-essential nutrients such as nitrogen can further enhance growth [9]. In this work, wheat bran was supplemented with a variety of nitrogen sources to determine whether phytase activity could be enhanced. A three-fold increase up to ~300 SPU/g was observed when adding LB broth at 10% compared with the un-supplemented control (Figure 2). Additive levels in excess of 10% were associated with decreased phytase activity, with an addition level of 25% causing a reduction of phytase activity below that of un-supplemented bran. Overabundance of nitrogen has been shown to reduce the production of hydrolytic enzymes due to excess cell biomass [10]. When the components of LB broth (*i.e.*, yeast extract, tryptone) were added individually, phytase production increased compared with the control, but was numerically less ($p > 0.05$) than that achieved with LB broth. When evaluating nutrient addition to any commercial scale fermentation, cost is an important factor to consider in relation to the added benefit of enzyme production. While LB is more expensive compared to any other nitrogen-based growth medium ingredient; LB was included in this study because it: (a) represents a readily recognized, and commercially available form of the two other nitrogen-rich media components used in this study; (b) LB is one of the most commonly used media ingredient for culturing *E. coli* under experimental conditions; and (c) on a large commercial scale, the ingredients that make up LB are readily available for a far more sensible price that laboratory qualities of the branded products. Our estimates are that the increase enzyme yield outstrip the increase in nutrient costs.

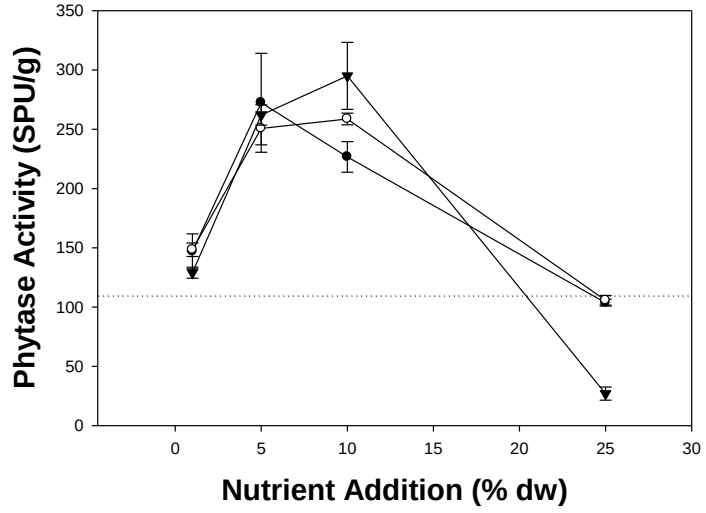

Figure 2. Effect of nitrogen-rich nutrient supplementation on phytase activity by *E. coli* during solid-state fermentation (SSF). ● Yeast extract; ○ Tryptone; and ▼ LB Broth powder were added at the concentration indicated. The dotted line represents phytase activity using unsupplemented wheat bran (control). Data shown are the averages and standard deviation (error bars) of three independent samples.

3.2. Effect of Moisture Level and Water Activity on Phytase Production

Moisture and water activity have been shown to be critical physiological parameters for enzyme production with relatively small reductions to water values having a marked negative influence on production [11]. Typical levels of substrate moisture levels for SSF enzyme production using fungi range from 20% to 70% (w/v). In comparison, bacterial growth typically requires moisture levels of approximately 70% [12]. The SSF bed moisture levels herein ranged from 40% to 80% (w/v). The poorest phytase activity (*i.e.*, 73 SPU/g phytase) was obtained at the 40% moisture level, whereas the maximum phytase yield of 362 SPU/g phytase was achieved at the 70% moisture level, closely followed by a yield of 309 SPU of phytase at the 60% moisture level (Figure 3). These moisture levels meet the definition of solid-state fermentation: microbial growth on solid particles in the absence of free water. At 60% to 70% moisture, the water present in SSF systems exists in a complexed form within the solid matrix or as a thin layer either absorbed to the surface of the particles or less tightly bound within the capillary regions of the solid. Free water becomes present only after the saturation capacity of the solid SSF matrix is exceeded [13].

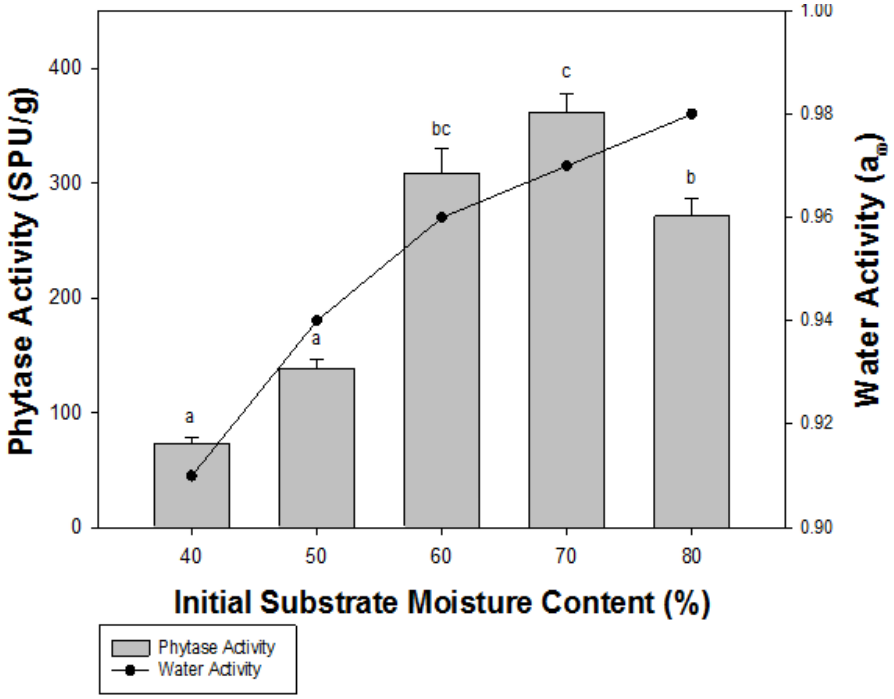

Figure 3. Effect of substrate moisture level on *E. coli* phytase activity during SSF on wheat bran. Flasks were incubated for 96 h at 37 °C with a relative humidity of 90%. The data reported are the average and standard deviation of three independent samples. Columns with different superscript letters differ significantly ($p < 0.05$.).

The roles of water in biological systems are numerous and have a significant impact on growth rates as discussed by Gervais and Molin [14]. The availability of water for biological reactions, especially expressed as water activity (a_ω), is directly correlated with growth rate. Water activity is defined as the ratio of vapor pressure of a liquid solution to that of pure water at the same temperature. Substrate water-binding properties can affect water availability. Many studies have addressed the importance of

maintaining water activity during fermentation and its effects on enzymatic stability, microbial growth, and enzyme expression [15]. The highest phytase production occurred herein for a_ω in the range of 0.96 to 0.97 (Figure 3).

3.3. Effect of Inoculum Rate on Phytase Production

Of the tested *E. coli* inoculum rates (ranging from 9.07×10^5 to 4.54×10^7 CFU g^{-1} bran), optimum phytase activity was achived from 2.1×10^7 to 1.1×10^7 CFU g^{-1} bran (Figure 4). While a decrease in inoculum rate from 4.5×10^7 to 2.27×10^7 was associated with an increase in phytase activity, further decreases in inoculum rate were associated with a decline in phytase activity. Effects of the inoculum rates on hydrolytic efficiency are known to vary between species and even strains of the same species [6].

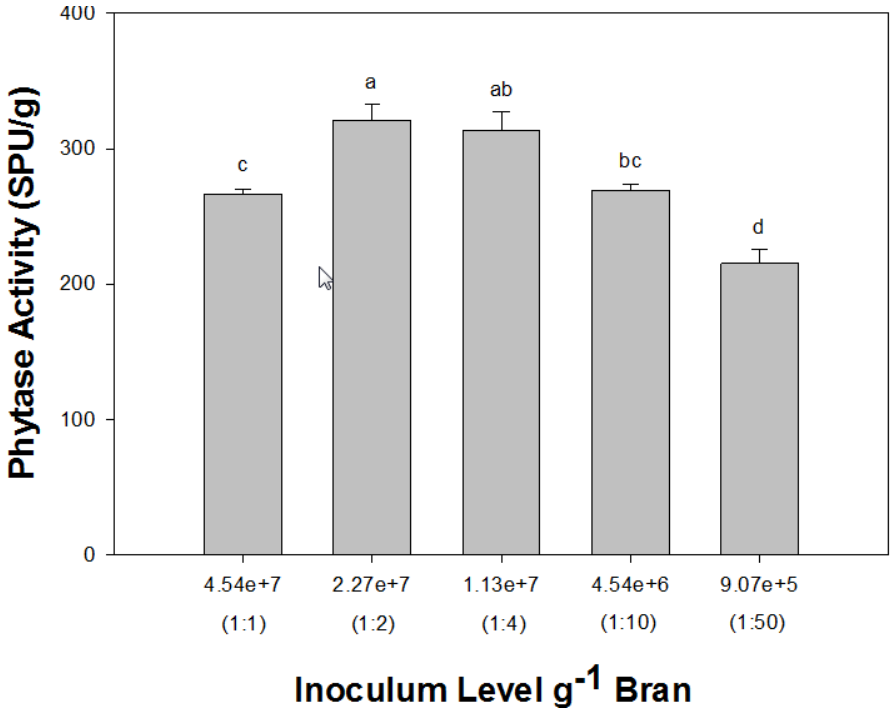

Figure 4. Effect of *E. coli* inoculant rate on phytase activity. Effect of inoculant level with sterile water and 8 h inoculum on phytase activity during SSF on wheat bran at 60% (*w/v*) moisture. Flasks were incubated for 96 h at 37 °C with a relative humidity of 90%. The data reported are the average and standard deviation of three independent samples. Columns with different superscript letters differ significantly ($p < 0.05$).

3.4. Effect of Incubation Period on Phytase Production

The period required to achieve optimal enzyme yield is of great economic importance. Shorter incubation periods translate into faster turnaround times between batches, shorter opportunity for spoilage, and lower operating cost required to maintain culture conditions (e.g., temperature). Over the 168 h period monitored herein, phytase activity was greatest (*i.e.*, 380 ± 10 SPU/g) after 96 h and remained relatively stable (Figure 5). In comparison, maximum enzyme production from fungal growth generally requires up to 144 h [16].

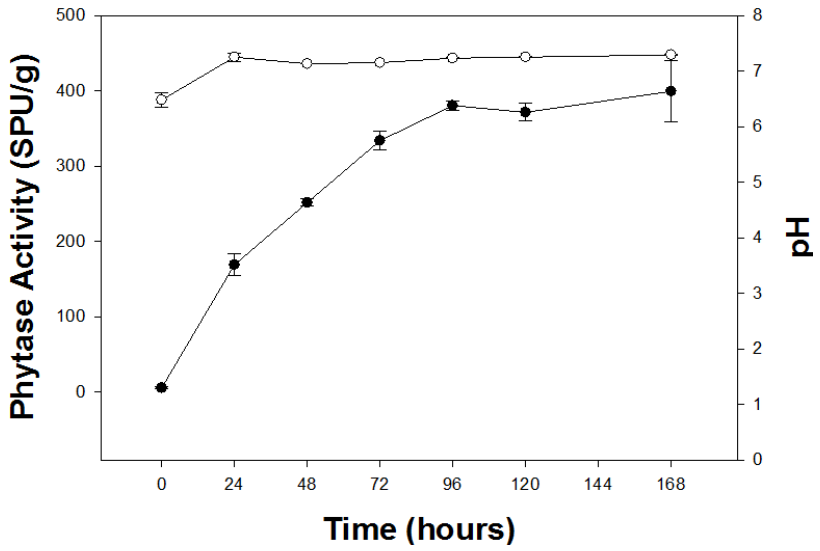

Figure 5. Effects of *E.coli* incubation time and pH on phytase activity. Effect of SSF incubation period on phytase activity sampled every 24 h between 0–168 h. The flask contained wheat bran moistened with a 24 h inoculum and sterile water at a ratio of 1:4. ○ pH; ● phytase activity (Solid State Fermentation Phytase unit (SPU) is defined as the amount of enzyme that will liberate 1 μmol of inorganic phosphate per minute at pH 5.5 °C and 37 °C). The data reported are the average and standard deviation of three independent samples.

3.5. Comparison between SmF and SSF on Phytase Productivity

Applying SSF to facilitate the production of phytase yields a maximum phytase activity of 350 ± 50 SPU per gram (Figures 2 and 3). This in itself represents a significant improvement compared to the control, which achieved a phytase yield of approximately 110 SPU per gram (Figure 1). In a final comparison for the application of a bacterial SSF application that employs *E. coli* as the fermentative organism for the production of phytase, we undertook a SmF fermentation with *E. coli* in a shake flask culture at 5% LB broth. We obtained our highest yield of phytase activity (64.5 SPU/g) within two days of incubation (no further data shown). Hence, the least optimised SSF system yielded approximately twice as much phytase activity compared to our best yield in SmF, while the optimised SSF conditions yielded a more than five-fold increase in phytase activity.

3.6. Effect of Temperature and pH on Phytase Activity

The phytase produced by *E. coli* under optimal conditions (70% (w/v) moisture, 1.13×10^7 CFU *E. coli* g^{-1} of bran, 10% LB broth powder, for 96 h at 37 °C) was assessed for stability and activity under various pH and temperature profiles using 2 g of dried SSF product. It is of importance that the phytase produced by this process will be able to withstand both the post-fermentation process and remain active in the digestive system of monogastric animals. Most feed is pelletized, which occurs at elevated temperatures; while the intestinal pH various between 3 and 6. The effect of pH and temperature on phytase activity was monitored from pH 2.5 to pH 7.5, and for temperatures ranging from 20 °C to 70 °C. Optimal phytase activity occurred at pH 5.5 and 50 °C (Figure 6). This activity was the highest

at pH 5.5 throughout the temperature profile. A broad range of optimal pH and temperature values for phytase activity has been reported in the literature across microbial species [17]. The optimal conditions displayed in Figure 5 are consistent with other studies of *E. coli* [7].

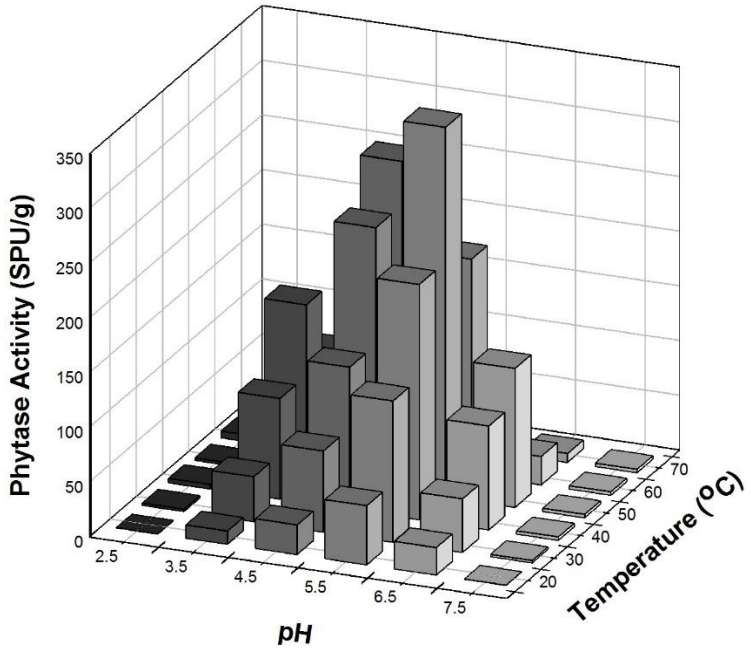

Figure 6. *E. coli* phytase activity optimization. The temperature and pH profile of phytase enzyme activity at various temperature and pH conditions. The temperature range was between 20 and 70 °C. The pH range was between 2.5 to 7.5.

3.7. General Discussion

Our results show that the application of SSF for the production of phytase by *E. coli* provides a marked improvement in yield in phytase activity over submerged cultivation (SmF). Under SSF conditions, a maximum phytase activity of 350 ± 50 SPU per gram of bran was achieved by incubating *E. coli* (2.27×10^7 CFU g^{-1}) on a solid substrate of wheat bran supplemented with 10% LB powder at 70% (*w/v*) moisture at 37 °C for 96 h. The phytase activity achieved under these conditions was 3.5 fold higher than the activity achieved under the least optimal conditions tested. Comparing our results to previous studies, it is clear that the microbial source plays a major factor in the conditions for maximum phytase activity. Typically, fungal SSF requires longer incubation periods up to 168 h, which presents challenges for contamination and increased operating cost. Previous bacterial SSF studies evaluating phytase have predominantly focused on *Bacillus* sp., which have shown similar results to the present study. Our findings are similar to those described for *Bacillus* sp. Which indicate an incubation time of 72–96 h, with improved results after nutrient supplementation [6,18,19].

4. Conclusions

The results in the present study suggest that bacterial phytase production utilizing *E. coli* on SSF technology is technically feasible, possibly offering a new, low-cost opportunity to produce a highly stable phytase as an alternative to *Bacillus* sp for bacterial SSF. Additional studies are under way to

evaluate phytase production by *Bacillus subtilis* and also a mixed *E. coli*/*B. subtilis* culture utilizing a wheat bran substrate.

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Author Contributions

Authors Kyle McKinney, Keith Filer, Andrea Humphries and Frank Vriesekoop contributed to the conception and design of the experiments; authors Kyle McKinney, Justin Combs, and Patrick Becker performed the experiments; while all authors were involved in the analyses of the data and contributed to the writing of the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

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