Media Evaluation for the Production of Microbial Enzymes†

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This study compares the performances of animal-based byproducts with other complex nutrients. Productions of α-amylase by *Bacillus subtilis*, glucoamylase by *Aspergillus niger*, endocellulase, exocellulase, and xylanase by *Trichoderma viride*, protease by *B. subtilis*, and *B. licheniformis* were evaluated in shake-flask fermentation. Culture broths were analyzed daily for enzyme activity for up to 9 days. Significant increases in α-amylase, cellulase, and xylanase activities were observed. Data strongly suggest that many of these byproducts can be used to replace the more expensive complex nutrients in industrial enzyme production with a corresponding increase in enzyme production.

Keywords: *Enzyme production; α-amylase; glucoamylase; endocellulase; exocellulase; xylanase; protease*

INTRODUCTION

Microbial fermentation is one of the most rapidly growing industries in the United States. Examples are commodity chemicals such as ethanol for gasoline extenders and oxygenators, lactic acid for degradable plastics (polylactic acid), amino acids for feed and food supplements, citric acid for pharmaceuticals and beverages, enzymes for detergents, the food industry, and the fermentation industry, succinic acid for foods, pharmaceuticals, cosmetics, lacquers, and dyes, and other oxychemicals. Other fermentation products are driving this growth (Datta et al., 1995). Culture medium supplies the microorganism with all of the essential elements for growth. Certain microorganisms are capable of biosynthesizing all of their cellular constituents from glucose and ammonium sulfate. However, most industrial microorganisms require some source of micronutrients (i.e., amino acids, trace elements, vitamins, and nucleic acids).

Fermentation medium can represent almost 30% of the cost for a microbial fermentation, with micronutrients representing the most significant cost of production (Miller and Churchill, 1986). Byproducts can supply unique micronutrients to replace expensive peptone and yeast extract. Shake-flask cultures are very useful in screening many media for target product formation in relatively short periods.

Consistency of ingredients used in commercial medium formulations and a significant increase in product yield or cost reduction are critical for industrial fermentation utilization of any byproduct. This paper addresses one of these issues, that is, the benefit to commercial enzyme yields. Significant increases in α-amylase, cellulase, and xylanase activities were observed when select byproducts were used to replace more expensive complex nutrients.

MATERIALS AND METHODS

Microorganisms. All cultures were obtained from the American Type Culture Collection (Rockville, MD). *Bacillus subtilis* (ATCC 21770), *Aspergillus niger* (ATCC 13497), *Trichoderma viride* (ATCC 32086), and *Bacillus licheniformis* (ATCC 21424) were maintained on nutrient agar (Difco Laboratories, Detroit, MI), potato dextrose agar (Difco), malt extract agar (Difco), and trypticase soy agar (Becton Dickinson, Cockeysville, MD), respectively, and stored as freeze-dried cultures and/or stock slant cultures at 4 °C. Incubation was performed by shaking at 125 rpm at 30 °C except for *B. licheniformis*, which was incubated at 37 °C.

Media. Animal-based byproducts (American Protein Corp., Ames, IA) were used as complex nutrients: pork stock, low degree of hydrolysis (DH) hydrolyzed beef fibrin (AP1130), intermediate DH hydrolyzed beef fibrin (AP1135), hydrolyzed beef serum concentrate (AP5135), beef stock, pork hydrolysate (AP100), pork hydrolysate (AP210), SD chicken broth (AP1020), hydrolyzed pork protein (AP5020), hydrolyzed beef protein (AP5030), pork hydrolysate (AP210-1). Each byproduct was substituted in equal concentration for major nitrogenous component(s) in the original media. All components and substituted nutrients for each microorganisms are designated in boldface type in the captions of Figures 1−7. After medium autoclaving, all cultures were grown in 250-mL Erlenmeyer flasks containing 100 mL of culture medium. Culture flasks were incubated at the culture's specific temperature with shaking (125 rpm).

Enzyme Assay. To determine enzyme activity, each culture broth was aseptically sampled (10 mL) daily for up to 9 days. Each sample was centrifuged at 3000*g* for 10 min, and supernatant was evaluated for extracellular enzyme activity. Upon addition of specific substrate, each enzyme activity was determined by following product formation at 0, 15, 30, and 60 min except for exocellulase activity, which was measured once after 20 h of incubation with shaking (250 rpm). Enzyme activity was expressed as micro- or milligrams of product

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produced per minute except for that of exocellulase, which was expressed as milligrams of glucose per 20 h. Shake-flask cultures for each medium combination for each culture were performed in replicates of three.

Reducing Sugar Assay. Polysaccharide hydrolase activity for α-amylase, glucoamylase, endocellulase, exocellulase, or xylanase was measured by determining the reducing sugars (maltose, glucose, and xylose) produced after addition of the corresponding substrate. The concentrations of reducing sugars released were assayed according to the dinitrosalicylic acid (DNS) method (Miller et al., 1960) after 0, 15, 30, and 60 min of incubation for each specific enzyme reaction mixture and then determined spectrophotometrically at 575 nm by using a Spectronic 20 (Milton Roy, Rochester, NY). Maltose was used as a standard for α-amylase (0−1600 μg of maltose/reaction tube with $r = 0.999$), glucose for glucoamylase, endocellulase, and exocellulase (0−1000 μg of glucose/reaction tube with $r = 0.995$), and xylose for xylanase (0−1400 μg of xylose/reaction tube with $r = 0.991$).

Polysaccharide Enzyme Assay Conditions. *α-Amylase.* α-Amylase (1,4-α-D-glucan-glucanohydrolase; EC 3.2.1.1) activity was measured at pH 6.9 and 20 °C. The reaction mixture contained 200 μL of crude cell-free extract, 7.8 mL of 0.1 M sodium phosphate buffer, and 12 mL of 3 g/L native corn starch solution (American Maize Products Co., Hammond, IN) as substrate (Fogarty, 1983). Final reaction solution contained 0.04 M sodium phosphate and 36 mg of native corn starch.

Glucoamylase. Glucoamylase (amyloglucosidase; exo-1,4-α-glucosidase; EC 3.2.1.3) was assayed at pH 4.5 and 55 °C. The reaction mixture consisted of 100 μL of culture supernatant, 7.9 mL of 0.06 M sodium acetate buffer, and 12 mL of 3 g/L native corn starch (American Maize Products Co.) solution as substrate (Fogarty, 1983). Final reaction solution contained 0.024 M sodium acetate and 36 mg of native corn starch.

Endocellulase. Endocellulase [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4] activity was measured at pH 5.0 and 37 °C. The reaction mixture contained 600 mL of culture supernatant, 7.4 mL of 0.06 M sodium acetate buffer, and 12 mL of 3 g/L carboxymethylcellulose (CMC) (Sigma, St. Louis, MO) solution as substrate (Wood and Bhat, 1988). Final reaction solution contained 0.023 M sodium acetate and 36 mg of CMC.

Exocellulase. Exocellulase (1,4-β-D-cellobiosidase; EC 3.2.1.91) was assayed at pH 5.0 and 30 °C. The reaction mixture contained 600 mL of culture supernatant, 1.0 mL of 10 g/L Sigmacell 20 (Sigma) cellulose solution as substrate, and 1.4 mL of 0.06 M sodium acetate buffer and was shaken in a test tube at 250 rpm for 20 h (Wood and Bhat, 1988). Final reaction solution had 0.035 M sodium acetate and 10 mg of cellulose.

Xylanase. Xylanase (1,4-β-D-xylanxylanohydrolase; EC 3.2.1.8) was assayed at pH 4.5 and 30 °C. The reaction mixture contained 300 mL of culture supernatant, 3 mL of 6 g/L xylan as substrate, and 6.7 mL of 0.06 M sodium acetate buffer (pH 4.5) (Steward and Heptinstall, 1988). Final reaction solution contained 0.041 M sodium acetate and 18 mg of xylan.

Protease Assay. The enzyme reaction mixture contained 1.0 mL of crude cell-free culture supernatant, 1.0 mL of 66.7 mM phosphate buffer (pH 7.5), and 2 mL of hemoglobin mixture, which was prepared by mixing 10 mL of 22% (w/v) hemoglobin solution (Sigma), 72 mL of deionized water containing 36 g of urea, and 8 mL of 1 N NaOH. After standing for 30 min at 25 °C, 10 mL of a solution of 1 M KH_2PO_4 containing 4 g of urea was added to the final solution (pH 7.5) (Anson, 1939). To each reaction mixture (0.75 mL) was added 1.25 mL of 0.3 N trichloroacetic acid, and the mixture was filtered by syringe-disk filter (0.2 μm; HT Tuffryn polysulfone membrane, Gelman Sciences Inc., Ann Arbor, MI). Finally, 1.0 mL of filtrate was mixed with 2.0 mL of 0.5 N NaOH solution, followed by 0.6 mL of 0.67 N phenol reagent (Sigma) addition with 10 min of reaction. Tyrosine production in the reaction mixture was determined spectrophotometrically at 750 nm at 0, 15, 30, and 60 min intervals (Ward, 1983).

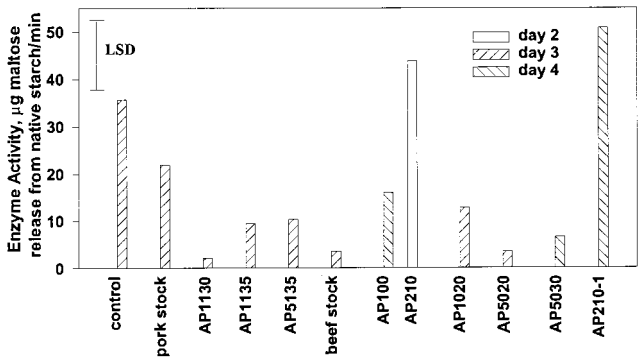

Figure 1. Maximum α-amylase activity (μg of maltose/min) produced by *B. subtilis* in shake-flask culture at 30 °C. Specific culture medium was 0.50% starch, 0.56% NH_4NO_3, 0.28% sodium citrate, 0.13% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, **0.05% peptone**, and **0.20% yeast extract**. Boldfaced ingredients were substituted in equal concentration. Each value is the average of three replicates. Significance level from the analysis of variance is $p = 0.0001$.

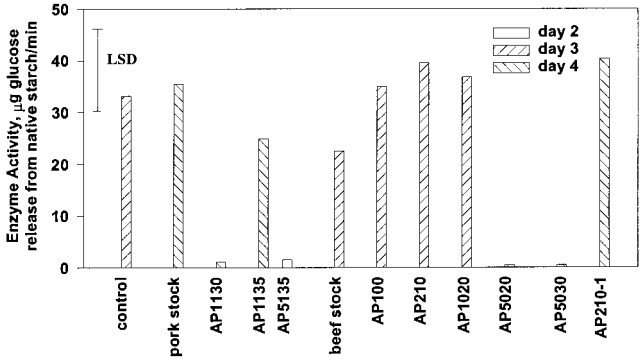

Figure 2. Maximum glucoamylase activity (μg of glucose/min) produced by *A. niger* in shake-flask culture at 30 °C. Specific culture medium was 0.5% maltose, **6.0% soy milk**, 7.0% sodium citrate, 1.5% $(NH_4)_2SO_4$, 0.1% NaH_2PO_4, 0.1% $MgSO_4$, and 0.1% Tween 80. Boldfaced ingredient was substituted in equal concentration. Each value is the average of three replicates. Significance level from the analysis of variance is $p = 0.0001$.

Statistical Analysis. Experimental error variance was estimated by analyzing the maximum enzyme activity data by use of analysis of variance for a completely randomized design using Statistical Analysis System package (version 6.09) (SAS Institute, Cary, NC). A 5% least significant difference (LSD) was used to determine significant differences between any two treatment means. Overall significance level (p value) among culture media from the analysis of variance is presented in each figure caption. Determinations were made in replicates of three.

RESULTS AND DISCUSSION

Various enzyme productions by specific microorganisms in modified production media were performed for 2−9 days in shake-flask cultures. Enzyme activity was determined during the fermentation for every 24 h. Samples with maximum activity were compared with controls.

α-Amylase. AP210-1 demonstrated highest *B. subtilis* α-amylase activity, which was 50.8 μg of maltose/min (Figure 1). Activities for AP210 and for the control

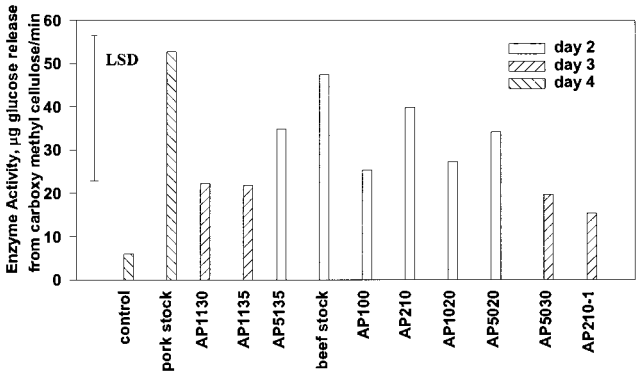

Figure 3. Maximum endocellulose activity (μg of glucose/min) produced by *T. viride* in shake-flask culture at 30 °C. Specific culture medium was 5.0% carboxymethylcellulose, 0.20% KH_2PO_4, 0.14% $(NH_4)_2SO_4$, **0.03% urea**, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2$, 5.0 ppm of $FeSO_4 \cdot 7H_2O$, 1.6 ppm of $MnSO_4 \cdot H_2O$, 1.4 ppm of $ZnSO_4$, and 2.0 ppm of $CoCl_2$. Boldfaced ingredient was substituted in equal concentration. Each value is the average of three replicates. Significance level from the analysis of variance is $p = 0.25$.

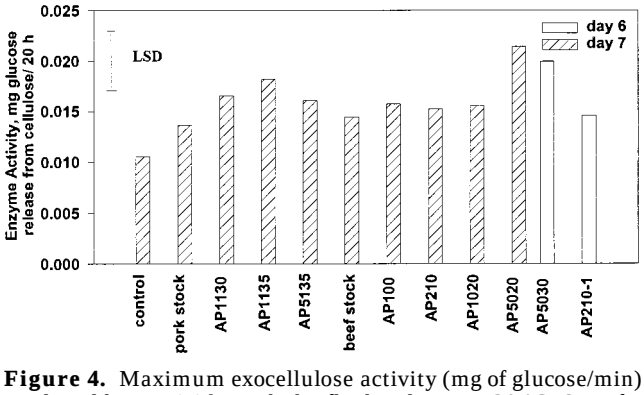

Figure 4. Maximum exocellulose activity (mg of glucose/min) produced by *T. viride* in shake-flask culture at 30 °C. Specific culture medium was 5.0% carboxymethylcellulose, 0.20% KH_2PO_4, 0.14% $(NH_4)_2SO_4$, **0.03% urea**, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2$, 5.0 ppm of $FeSO_4 \cdot 7H_2O$, 1.6 ppm of $MnSO_4 \cdot H_2O$, 1.4 ppm of $ZnSO_4$, and 2.0 ppm of $CoCl_2$. Boldfaced ingredient was substituted in equal concentration. Each value is the average of three replicates. Significance level from the analysis of variance is $p = 0.11$.

were 43.7 and 35.7 μg of maltose/min, respectively. These activities were not significantly different (LSD of 16.8 μg of maltose/min). However, the activities of AP210-1 and AP210 were significantly different from those of the other byproducts evaluated with pork stock illustrating the next highest enzyme activity (21.9 μg of maltose/min). AP210 demonstrated maximum enzyme activity on day 2, whereas for the control and for AP210-1 maximum activity occurred on days 3 and 4, respectively. Decreased fermentation times can result in significant cost savings to the industry. The media may be grouped as AP210-1, AP210, control < pork stock < AP100, AP1020, AP5135, AP1135, AP5030, beef stock, AP5020, AP1130.

Glucoamylase. *A. niger* maximum glucoamylase activity was detected on day 2 and demonstrated maximum activity on days 3 and 4 of the fermentation (Figure 2). AP210-1 and AP210 again demonstrated the highest activities (40.4 and 39.6 μg of glucose/min, respectively), but these were not significantly different from those AP1020, pork stock, AP100, control, and AP1135 (LSD of 16.4 μg of glucose/min). Maximum activity for AP210-1 occurred on day 4, whereas for AP210 and control it occurred on day 3. Considering the fact that maximum activities for AP210-1 and AP210 were not significantly different, AP210 reaching the maximum level on day 2 makes it a better choice. The rest of the media, except beef stock, produced very low enzyme activities that were significantly different from the activity values already mentioned. All media may be grouped for *A. niger* glucoamylase activities as AP210-1, AP210 < AP1020, pork stock, AP100, control, AP1135, beef stock < AP5030, AP5135, AP1130, AP5020.

Endocellulase. All of the byproducts demonstrated higher endocellulase activities for *T. viride* than the control (Figure 3). Although pork stock demonstrated the greatest activity (52.7 μg of glucose/min), it was not significantly different from the other byproducts (LSD of 33.7 μg of glucose/min). However, pork stock, beef stock, and AP210 were significantly different from the control. Pork stock had maximum activity on day 4, whereas beef stock and AP210 illustrated maximum activity on day 2 of the fermentation (47.4 and 39.8 μg of glucose/min, respectively). Moreover, cell growth by this filamentous microorganism was more rapid on byproducts, which was indicated by green pigment and low viscosity. The byproduct substitution for this culture medium was 0.03%, yet this small substitution triggered significant increases in endocellulase production. Possibly some trace elements in these products enhanced enzyme biosynthesis. All media may be grouped for *T. viride* endocellulase activities as pork stock, beef stock AP210 < AP5135, AP5020, AP1020, AP100, AP1130, AP1135, AP5030, AP210-1, control (Figure 3).

Exocellulase. *T. viride* exocellulase activity was followed from days 4 to 9 (Figure 4). Generally, enzyme activity peaked on days 6 and 7. Because of the slow enzyme activity on the substrate (Sigma cellulose), enzyme reaction mixtures were incubated for 20 h with shaking. Results were reported as milligrams of glucose per 20 h. There were some significant differences among the various media (LSD of 0.0063 mg of glucose/20 h). AP5020 demonstrated greatest activity (0.0214 mg of glucose/20 h) on day 7, whereas for the control maximum activity occurred on day 6 (0.0105 mg of glucose/20 h). All media may be grouped for *T. viride* exocellulase activities as AP5020, AP5030, AP1135, AP1130, AP5135, AP100, AP1020, AP210, AP210−1, beef stock, pork stock < control.

Xylanase. *T. viride* xylanase activity was followed from days 5 to 7 (Figure 5). Xylanase from *T. viride* showed trends similar to those demonstrated in endocellulases and exocellulases. All byproducts performed better than the control. AP5135 demonstrated highest activity on day 6 (0.198 mg of xylose/min). However, this was not significantly different from AP1135, AP1130, beef stock, AP210, and AP1020, but it was significantly different from the other byproducts and the control (LSD of 0.092 mg of xylose/min).

Protease. *B. subtilis* protease activity was highest for AP210-1 (2.13 μg of tyrosine/min) for day 3, followed by AP100, beef stock, and AP1020 (1.90, 1.72, and 1.50 μg of tyrosine/min, respectively), whereas the control demonstrated 1.48 μg of tyrosine/min for day 2 (Figure 6). With the calculated LSD (0.81 μg of tyrosine/min), the protease activities of AP210-1, AP100, beef stock, and AP1020 were not significantly different from that of the control (1.48 μg of tyrosine/min). The only difference between them was that the control reached

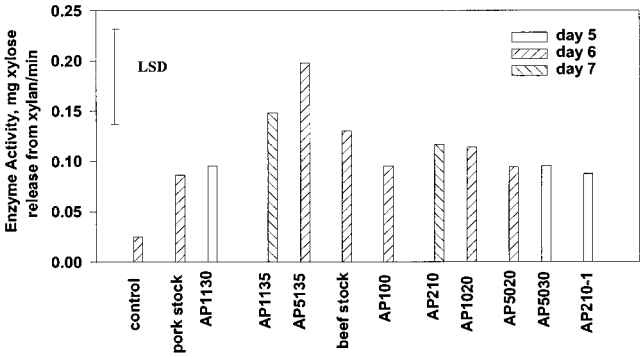

Figure 5. Maximum xylanase activity (mg of xylose/min) produced by *T. viride* in shake-flask culture at 30 °C. Specific culture medium was 5.0% carboxymethylcellulose, 0.20% KH_2PO_4, 0.14% $(NH_4)_2SO_4$, **0.03% urea**, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2$, 5.0 ppm of $FeSO_4 \cdot 7H_2O$, 1.6 ppm of $MnSO_4 \cdot H_2O$, 1.4 ppm of $ZnSO_4$, and 2.0 ppm of $CoCl_2$. Boldfaced ingredient was substituted in equal concentration. Each value is the average of three replicates. Significance level from the analysis of variance is $p = 0.12$.

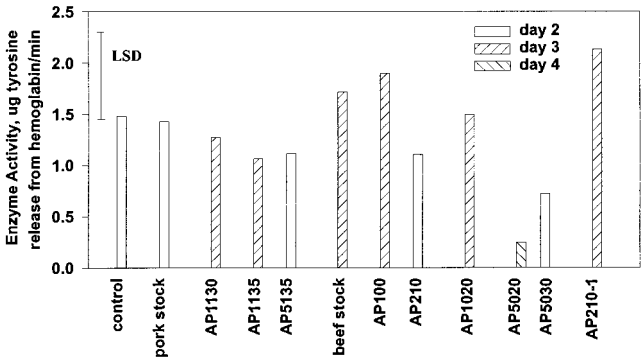

Figure 6. Maximum protease activity (μg of tyrosine/min) produced by *B. subtilis* in shake-flask culture at 30 °C. Specific culture medium was 2.00% starch, 0.56% NH_4NO_3, 0.28% sodium citrate, 0.13% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, **0.05% peptone**, and **0.20% yeast extract**. Boldfaced ingredients were substituted in equal concentration. Each value is the average of three replicates. Significance level from the analysis of variance is $p = 0.006$.

maximum activity on day 2, whereas the mentioned byproducts reached maximum activity on day 3. The media may be grouped for *B. subtilis* protease activity as AP210-1, AP100, beef stock, AP1020, control, pork stock > AP1130, AP5135, AP210, AP1135 < AP5030, AP5020.

AP1130 on day 2 had the highest *B. licheniformis* protease activity (3.49 μg of tyrosine/min), which was significantly different from that of the control (2.04 μg of tyrosine/min) and the remaining byproducts (LSD of 0.78 μg of tyrosine/min) (Figure 7). The control demonstrated 2.04 μg of tyrosine/min, which was not significantly different from that of AP5135, AP1135, or pork stock. All media may be grouped for *B. licheniformis* protease activities as AP1130 < AP5135, control, AP1135, pork stock > beef stock, AP100, AP1020, AP5030, AP210-1, AP210 >AP5020.

Conclusions. These data strongly suggest that many of these natural byproducts have a tremendous potential to benefit the industrial enzyme industry by increased enzyme production. Further research is needed to evaluate enzyme production in controlled fermentors, the potential interference in enzyme recovery by these products, and the optimization of enzyme production in various medium blends.

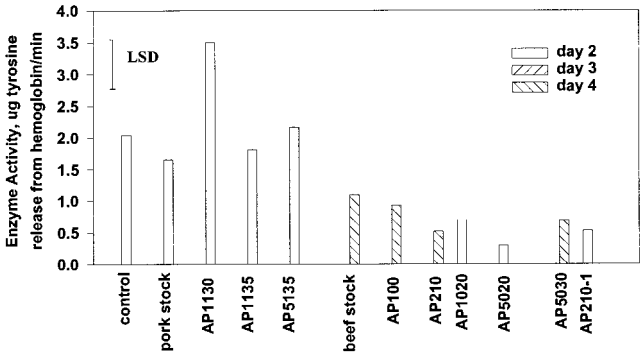

Figure 7. Maximum protease activity (μg of tyrosine/min) produced by *B. licheniformis* in shake-flask culture at 30 °C. Specific culture medium was 2% glucose, **2% soy flour**, 0.25% corn steep liquor, 0.5% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $FeSO_4 \cdot 7H_2O$, and 0.3% $CaCO_3$. Boldfaced ingredient was substituted in equal concentration. Each value is the average of three replicates. Significance level from the analysis of variance is $p = 0.0001$.

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