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Mathematical Modeling of the Development of Antioxidant Activity in Soybeans Fermented with *Aspergillus oryzae* and *Aspergillus awamori* in the Solid State

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The kinetics of the development fungal growth, β-glucosidase activity, total phenolics, and DPPH scavenging in soybeans fermented with *Aspergillus oryzae* and *Aspergillus awamori* were studied over an 8-day incubation period. Modified logistic equations were then used to describe the experimental fermentation profiles. In all cases the models were consistent and the parametric estimations were statistically significant. The predicted values have high coefficients of linear correlation with the experimental results, and the kinetic parameters obtained show that *A. oryzae* grows more rapidly and produces more antioxidant activity than *A. awamori*.

KEYWORDS: Antioxidants; soybeans; *Aspergillus oryzae*; *Aspergillus awamori*; solid state fermentation; logistic model

INTRODUCTION

Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have a restricted use in foods as they are suspected to have carcinogenic effects. For this reason the search for natural antioxidant sources has greatly increased in recent years (*1*). At present, one of the foods that has received considerable attention due to its production worldwide, high-quality oil, protein contents, and functional compounds is soybean (*2*). This legume contains various amounts of phytochemicals (isoflavones, saponins, phytic acid, phytosterols, Kunitz and Bowman−Birk trypsin inhibitors, and phenolic acids) that show functional, antioxidant, and radical scavenging properties (*2, 3*). Among these, phenolic compounds are one of the most important bioactive components. These molecules are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens (*4*). Their antioxidant activity is basically due to the quenching of oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radicals (*5*).

It has also been reported that the concentration of phenolics significantly increases after fermentation when compared to unfermented soybeans (*6*−*8*). For example, the antioxidant concentration was significantly higher in fermented soybean products such as miso, natto, tempeh, and koji (using *Aspergillus oryzae*, *Bacillus natto*, *Rhizopus oligosporum*, and *Rhizopus oryzae*, respectively, as starters) than in a nonfermented soybean (*9*). The antioxidant activities were also reported in submerged cultures of soybeans with different *Lactobacillus* and *Bifidobacteria* (*10*−*12*).

The genus *Aspergillus* is one of the most common fungus groups and has been associated for a long time with traditional food fermentations in oriental countries, particularly soy sauce, koji, and miso production. It is also an important source of industrial enzymes (*13*−*17*). Although some previous works have studied the antioxidant properties of fermented soybeans (*8,18*), a mathematical modeling approach to predict and simulate the development of antioxidants in these bioprocesses has not been applied before.

The objective of this work is to monitor the total phenolic content, DPPH scavenging effect, β-glucosidase activity, and microbial growth in soybeans fermented with *A. oryzae* and *Aspergillus awamori* over an 8-day incubation period. A set of modified logistic models was then used to fit the experimental results and predict the fermentation profiles. The significant kinetic parameters obtained allowed the characterization of product formation and the selection of *A. oryzae* as an appropriate fungus for increasing antioxidant activity in fermented soybeans.

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MATERIALS AND METHODS

Microorganisms. *A. awamori* 2B 361 U2/1 was obtained from ABM Chemicals Ltd. (Woodley, Cheshire, U.K.), which has been used for the commercial production of amyloglucosidase. *A. oryzae* was isolated from a soy sauce starter (*19*). The fungus spore suspensions were initially store in distilled water at −30 °C. The cell concentrations in these suspensions were 1.4×10^8 cells/mL for *A. awamori* and 1.2×10^8 cells/mL for *A. oryzae*, and the volume of inoculum in both cases was 1.5 mL.

Soybean Fermentation. Split soybeans (150 g) and 73.5 mL of distilled water were placed in 1 L capped Duran bottles and autoclaved at 121 °C for 20 min. The spore suspension was then mixed with the sterile medium, and the bottles were manually shaken (vertically and horizontally) for 10 min to homogenize the spores. The inoculated soybeans were poured into Petri dishes and incubated at 30 °C over 8 days. Two Petri dishes were collected every day, and the fermented soybeans were crushed with a mortar and pestle before being sealed in plastic bag and stored at −30 °C until used.

Spore Counting. Spore counting of the fresh fermented soybeans was determined microscopically using a hemocytometer with a 0.1 mm depth (Improved Neubauer, Weber, U.K.) under a magnification of 500. A fermented soybean suspension was prepared by placing 0.5 g of the sample and 3 mL of distilled water in a test tube and homogenizing for 10 s. Dilutions of the suspensions for spore counting were made when necessary.

Crude Enzyme Extraction. Three grams of ground sample was placed in a 100 mL Duran bottle and mixed with 15 mL of distilled water. The bottles were shaken at 150 rpm and 30 °C for 1 h in an orbital shaker, and then samples were centrifuged at 16250*g* and 4 °C for 10 min. Supernatants were filtered through a Whatman no. 1 filter paper and kept at −30 °C until used for the enzyme activity assay.

Determination of β-Glucosidase Activity. The β-glucosidase activity of fermented soybeans was estimated using the McCue and Shetty method (*7*). One hundred microliters of 9 mM *p*-nitrophenol-β-D-glucopyranoside was mixed with 800 μL of 200 mM sodium acetate buffer (pH 4.6) in a test tube. Tubes were incubated at 50 °C in a water bath for 5 min before the addition of 100 μL of crude enzyme extract and further incubated for 30 min. In the blank the extract was replaced by distilled water. One thousand microliters of 100 mM sodium carbonate was added to stop the reaction, and then the samples were centrifuged at 16250*g* for 1 min. The absorbance of *p*-nitrophenol released was measured at 400 nm. The units of enzyme activity were defined (U/mg) as the micrograms of *p*-nitrophenol released in 1 min under controlled conditions.

Phenolics Extraction. Phenolic compounds from 2 g of ground samples were extracted with 20 mL of methanol using the Soxtec System HT (1043 Tecator) for 1 h at 60 °C. The extract was dehydrated to obtain a dry extract that was then diluted with methanol to make a 20 mg/mL extract suspension. This solution was centrifuged at 16250*g* for 5 min, and the supernatant was used for the determination of antioxidant properties.

Determination of Total Phenolics Content. The total phenolics assay was based on the Singleton et al. method (*20*) using Folin−Ciocalteu reagent (FCR) with gallic acid as a standard. A sample or methanol blank (50 μL) was added to 3 mL of distilled water in 15 mL test tubes. A volume of FCR (250 μL) was placed into the tube and mixed. Saturated Na_2CO_3 (750 μL) was added, and the total volume was adjusted to 5 mL with distilled water. The absorbance of the sample at 765 nm in a 1 cm cuvette was read after incubation for 2 h at room temperature. Readings were compared to the standard curve of gallic acid, and the total phenolics content was expressed as milligrams of gallic acid equivalents per gram of dry base of fermented soybeans (mg of GAE/g of db).

Determination of DPPH Radical Scavenging Activity. The effect of the extract on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was estimated according to the procedure described by Brand-Williams et al. (*21*). Phenolics extract (0.1 mL) was added to 3.9 mL of DPPH 6×10^{-5} M in methanol that was prepared daily. The decrease in absorbance was determined at 515 nm after incubation for 30 min. The DPPH solution without sample but with 0.1 mL of methanol was used as control and the inhibitory percentage of DPPH calculated according to the following equation:

$$\text{DPPH scavenging effect (\%)} = \left(1 - \frac{\text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}}\right) \times 100 \quad (1)$$

Numerical and Statistical Methods. Fitting procedures and parametric estimations calculated from the results were performed by minimization of the sum of quadratic differences between observed and model/equation predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro solver of the Microsoft Excel spreadsheet. Statistica 6.0 software (StatSoft, Inc., 2001) was used to evaluate the significance of the estimated parameters by fitting the experimental values to the proposed mathematical models and the consistency of these equations.

RESULTS

The purpose of this work was to obtain a formalized description of the cultures of two *Aspergillus* strains in a soybean medium. The maximum production of cellular biomass (*N*), β-glucosidase activity (*E*), total phenolic (*P*), and the highest DPPH scavenging activity (*D*) were characterized and predicted by the kinetic models. The aspergilli selected were used to produce antioxidative activities in previous fermentations carried out in submerged cultures using complex media (*22*) (data not shown).

Figure 1 shows the experimental data from the soybean solid state cultures fitted to the reparametrized logistic equation shown below (*23*−*27*). As can be observed, product formation improved in all cases with the fermentation process. In **Table 1** the notation and the dimensions of the parameters are summarized.

$$\ln\left(\frac{N}{N_0}\right) = \frac{K}{1 + \exp\left[2 + \frac{4\mu_m}{K} \times (\lambda - t)\right]} \quad \text{with } N_m = N_0\, e^K \quad (2)$$

A similar reparametrized model was employed to fit the antioxidant properties (total phenolic and DPPH scavenging) and the enzymatic production. Due to the presence of initial values of non-null activity, the equation was modified by introducing a non-null ordinate (Y_0).

$$Y = \frac{Y_1}{1 + \exp\left[2 + \frac{4\upsilon_{my}}{Y_1} \times (\lambda_y - t) + \frac{4Y_0}{Y_1}\right]} + Y_0 \quad (3)$$

In the previous equation *Y* represents the variable fitted with eq 3 (total phenolic, DPPH scavenging, and enzyme production). The limit of the function as time approaches infinity (when antioxidant production and enzymatic activity are maximum) gives

$$\lim_{t \to \infty} Y = Y_m = \frac{Y_1}{1 + \exp\left[2 + \frac{4\upsilon_{my}}{Y_1} \times (\lambda_y - \infty) + \frac{4Y_0}{Y_1}\right]} + Y_0 = Y_1 + Y_0 \quad (4)$$

Equation 3 was used to fit the experimental data of *E* (β-glucosidase activity), *D* (DPPH scavenging), and *P* (total phenolics). The values of the parameters Y_m, υ_{my}, and λ_y obtained in each case with confidence intervals are shown in **Table 2**.

A. oryzae led to higher and faster growth in soybeans (N_m = 4.96 cells/g and $\mu_{mn} = 2.29\ \text{day}^{-1}$). This strain also showed the

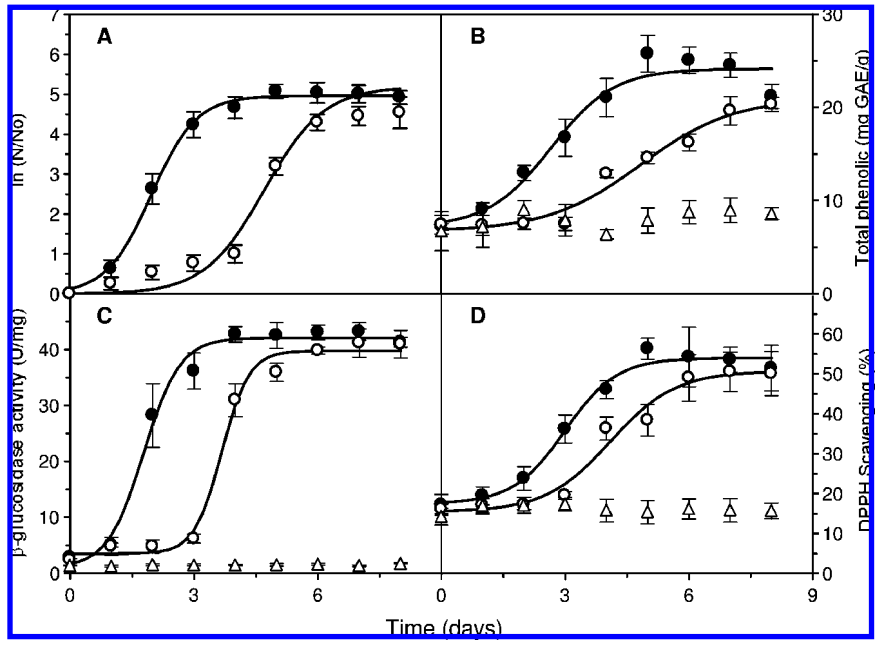

Figure 1. Kinetics of fungal growth (**A**), antioxidant production (**B**, total phenolics; **D**, DPPH scavenging) and β-glucosidase activity (**C**) in soybeans fermented with *A. oryzae* (●) and *A. awamori* (○), as well as control without fungi (△). Total phenolics were measured as gallic acid equivalents (mg/g of db). The error bars are the standard error of six measures (2 fermentation replicates × 3 independent analytical determinations).

Table 1. Notations Used with Units

N	cellular biomass; dimensions, cell number/g of db
N_0	initial cellular biomass; dimensions, cell number/g of db
t	time; dimensions, days
K	maximum relative cellular biomass; dimensionless
μ_{mn}	specific maximum growth rate; dimensions, day^{-1}
λ_n	growth lag phase; dimensions, days
N_m	maximum cellular biomass; dimensions, cell number/g of db
E_m	maximum enzymatic activity; dimensions, units/mg
V_{me}	maximum enzymatic production rate; dimensions, units mg^{-1} day^{-1}
λ_e	enzymatic activity lag phase; dimensions, days
P_m	maximum total phenolics; dimensions, mg of gallic acid equiv/g of dry base of soybeans (mg of GAE/g of db)
v_{mp}	maximum total phenolics production rate; dimensions, mg of GAE g^{-1} day^{-1}
λ_p	phenolics activity lag phase; dimensions, days
D_m	maximum DPPH scavenging activity; dimensions, percent
v_{md}	maximum DPPH inhibition rate; dimensions, percent/day
λ_d	DPPH activity lag phase; dimensions, days

highest production of antioxidant activities (P_m = 24.15 mg/g and D_m = 53.98%), maximum antioxidant production rate (v_{mp} = 5.58 mg g^{-1}day^{-1} and v_{md} = 14.54%/day), and the smaller antioxidant production lag phase (λ_d = 2.94 days).

In line with these results, the β-glucosidase activities of *A. oryzae* and *A. awamori* showed similar patterns (see **Figure 1**). The *A. oryzae* maximum enzymatic activity (E_m) was higher than *A. awamori* activity, 42.12 and 39.62 U/mg, respectively. However, the maximum rate of enzymatic production (v_{me}) and the lag phase (λ_e) of *A. oryzae* were lower than those of *A. awamori*. The control showed a more or less stationary value through time and was not fitted to the mathematical models.

In all cases, the equations proved to be consistent (Fisher's F; α = 0.05), and the parametric estimations (with the exception of λ_p) were statistically significant (Student's t; α = 0.05). All of the predicted values obtained by the nonlinear adjustments demonstrated high coefficients of linear correlation with the real observed values (see **Table 2**).

Table 2. Parametric Estimations (± CI; See **Table 1**) Corresponding to the Kinetic Models (2−4) Applied to Cultures of *Aspergillus oryzae* and *Aspergillus awamori* in Soybeans[a]

	A. oryzae	*A. awamori*
	Growth (M)	
K	4.963 ± 0.145	4.639 ± 0.614
μ_{mn}	2.292 ± 0.440	1.743 ± 0.811
λ_n	0.894 ± 0.234	3.234 ± 0.681
N_m	(1.66 ± 0.05) × 10^{10}	(1.39 ± 0.18) × 10^{10}
F (df_1 = 3; df_2 = 6; α = 0.05)	3085.30	238.06
p value	<0.0001	<0.0001
r (obs-pred)	0.9987	0.9927
	β-Glucosidase Activity (E)	
E_m	42.117 ± 2.504	39.620 ± 2.590
v_{me}	23.537 ± 12.551	27.451 ± 19.323
λ_e	0.950 ± 0.940	3.247 ± 0.537
F (df_1 = 4; df_2 = 5; α = 0.05)	581.82	469.48
p value	<0.0001	<0.0001
r (obs-pred)	0.9945	0.9959
	Total Phenolics (P)	
P_m	24.149 ± 2.848	21.010 ± 4.777
v_{mp}	5.580 ± 4.699	3.183 ± 1.868
λ_p	2.420 (NS)	4.689 ± 1.901
F (df_1 = 4; df_2= 5; α = 0.05)	239.68	317.08
p value	<0.0001	<0.0001
r (obs-pred)	0.9775	0.9859
	DPPH Scavenging (D)	
D_m	53.980 ± 3.275	50.659 ± 6.288
v_{md}	14.542 ± 7.160	11.206 ± 7.130
λ_d	2.941 ± 0.860	3.904 ± 1.312
F (df_1 = 4; df_2 = 5; α = 0.05)	811.99	315.52
p value	<0.0001	<0.0001
r (obs-pred)	0.9938	0.9878

[a] CI, confidence intervals (α = 0.05); F, F Fisher test (df_1, degrees of freedom for model; df_2, degrees of freedom for error); r, correlation coefficient between observed (obs) and predicted (pred) data; NS, not significant.

DISCUSSION

A number of analytical methods have been described for the evaluation of the antioxidant activity of natural compounds in food or biological systems. Due to the complexity of the

composition of foods, to separate and study individual antioxidant compounds would be costly and inefficient and would not consider the possible synergistic interactions among the antioxidants in a food matrix (*28*−*30*).

The Folin−Ciocalteu method has been applied for many years to measure total phenolics in natural sources. This method is based on an oxidation/reduction reaction and can be used for antioxidant determination (*31*). Sánchez-Moreno (*32*) suggested the DPPH assay to accurately measure the antioxidant capacity of fruit and vegetable juices or their extracts (*28*). Moreover, this test is simple and rapid, which probably explain its widespread use in antioxidant screening (*31*). On the basis of these considerations and due to the difficulty in associating antioxidant properties from complex matrix with only one or two specific compounds, the antioxidants produced from fermented soybeans in the solid state were measured by the total phenolics and the DPPH scavenging activity.

There are previous works reporting antioxidant properties in soybeans fermented with various inocula such as *Aspergillus* (*6*, *8*), *Rhizopus oligosporus* (*7*), *Saccaromyces cerevisiae* (*1*), and *Neurospora intermedia* (*33*). However, the use of models to describe antioxidant development during fermentation has not been done before, and comparisons with previous studies cannot be done. In recent years, artificial neural networks (ANN) have been broadly employed for the prediction of food processes (*34*−*36*). This mathematical tool, based on biological neural networks, uses adaptive models that change their structure as a function of external or internal information that flows through the network during the learning phase. However, these black boxes do not have an explicit equation formulated with real kinetic parameters to define properties of the fermentations. The logistic equation proposed in this work has a pseudomechanistic approach and has been widely used in the simulation, prediction, and control of fermentation processes (*26*, *37*). The parameters obtained from this model were biologically and statistically significant and allowed a comparison of product formation from both *Aspergillus* species.

Fermentation with *A. oryzae* achieved the maximum total phenolics concentration of 25 mg of gallic acid/g of dried soybeans after 5 days. A maximum of 20 mg of gallic acid/g of dried soybeans was obtained with *A. awamori* after 8 days. Lin et al. (*8*) found that the phenolics content of soybean koji with *A. awamori* increased between 18 and 45.7 mg of gallic acid/g of extract in 3 days and up to 42 mg of gallic acid/g of extract of the same koji with *A. oryzae*. Fernandez-Orozco et al. (*38*) also reported a phenolics concentration increment after 2 days of incubation (3.0−3.6 mg of catechin/g) in soybeans fermented with *A. oryzae*. The discrepancies could be due to the differences in the processing and fermentation techniques used and the methods used to quantify the antioxidant activity. Esaki et al. (*6*) utilized different strains of *Aspergillus* to ferment soybeans, and although they used similar experimental conditions, they reported different antioxidant properties for the different strains used.

The development of β-glucosidase activity of fermented soybeans was in line with the phenolics trend. It was suggested that in order to cleave phenolics from carbohydrate conjugates, fungal enzymatic activity was required (particularly from β-glucosidase) (*7*). The liberation of lipophilic aglycones of isoflavones glucosides by catalytic action of β-glucosidase during fermentation resulted in the enhancement of antioxidant activity in the miso and tempe processes (*8*). Miura et al. (*39*) reported an increasing β-glucosidase activity in soybean extract fermented with *Ganoderma lucidum* after 9 days, which was attributed to a decrease of isoflavone glycoside and the corresponding aglycone increment.

DPPH inhibition appeared to correlate with the total phenolics in our work, but no similar works are available in the literature for comparison. McCue and Shetty (*7*) reported no particular trend of DPPH radical activity in soybeans cultured with *R. oligosporus* for up to 10 days, and the DPPH scavenging effect was never higher than 30%.

In conclusion, the results of cellular biomass, enzymatic activity, phenolics content, and DPPH scavenging effect indicate that solid state fermentation of soybeans with *A. oryzae* for 5 days provided the best conditions for a maximum antioxidant activity production, and these conditions could be used in process development for natural antioxidant production.

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