Mathematical modelling of the development of antioxidant activity in soybeans fermented with *Aspergillus oryzae* and *Aspergillus awamori* in solid state

Dyah Hesti Wardhani¹, José Antonio Vázquez¹,², and Severino S. Pandiella¹*

¹School of Chemical Engineering and Analytical Science
The University of Manchester
Sackville Street, PO Box 88, Manchester M60 1QD, UK

²Grupo de Reciclado y Valorización de Materiales Residuales
Instituto de Investigaciones Marinas (CSIC)
r/ Eduardo Cabello, 6. Vigo-36208. Galicia – Spain

*Corresponding author E-mail: s.pandiella@manchester.ac.uk
Tel +44 (0)161 306 4429, Fax +44(0)161 306 4399.

Running title: Antioxidant activity in soybeans fermented by *Aspergilli*. 
Abstract

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 faster and produces more antioxidant activity than A. awamori.

Running title: Antioxidant activity in soybeans fermented by Aspergilli.

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). Recently, one of the food sources that have received an increasing attention due to their wide distribution around the world, high-quality oil, protein contents and functional compounds, is soybeans (2). These legumes contain various amounts of phytochemicals (isoflavones, saponins, phytic acid, phytosterols, Kunitz and Bowman-Birk trypsin inhibitors, phenolic acids) that show functional, antioxidants 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 the defense against ultraviolet radiation or aggression by pathogens (4). Their antioxidant activity is basically due to quench 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 instance, the antioxidant of fermented soybean products as miso, natto, tempeh and koji using as starters Aspergillus oryzae, Bacillus natto, Rhizopus oligosporum and Rhizopus oryzae, respectively, was significantly higher than in a non fermented 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 fungi groups and has been associated for a long time with traditional foods 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 simulated 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 *A. awamori* over an 8-day incubation period. A set of modified logistic models were then used to fit the experimental results and predict the fermentation profiles. The significant kinetic parameters obtained allowed the characterisation of these bioproductions and the selection of *A. oryzae* as an indicated fungi for increasing antioxidant activity in fermented soybeans.

**MATERIALS AND METHODS**

**Microorganisms**
Aspergillus awamori 2B. 361 U2/1 was obtained from ABM Chemicals Ltd. (Woodley, Cheshire, UK) which has been used for the commercial production of amyloglucosidase. Aspergillus oryzae was isolated from a soy sauce starter (19). The fungi spores suspensions were initially store in distilled water at -30°C. The cells concentration in these suspensions was 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.

Soybeans 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 minutes. The spore suspension was then mixed with the sterile medium and the bottles were manually shaken (vertically and horizontally) for 10 minutes to homogenise the spores. The inoculated soybeans were poured into petri dishes and incubated at 30°C over an 8-day period. Two petri dishes were collected everyday and the fermented soybeans were crushed with mortar and pestle before sealed in plastic bag and store at -30°C until used.

Spore Counting

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

**Crude enzyme extraction**

Three grams of ground sample were placed in a 100 ml Duran bottle and mixed with 15 ml of distilled water. The bottles were shaken at 150 rpm and 30ºC for 1 h in an orbital shaker, and then samples were centrifuged at 16,250×g and 4ºC for 10 minutes. Supernatants were filtered through a Whatman No 1 filter paper and kept at -30ºC 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). 100 μl of 9 mM p-nitrophenol-β-D-glucopyranoside were mixed with 800 μl of 200 mM sodium acetate buffer (pH 4.6) in a test tube. Tubes were incubated at 50ºC 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. 1000 μl of 100 mM sodium carbonate was added to stop the reaction, and then the samples were centrifuged at 16,250×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 number of p-nitrophenol μg released in one minute under controlled conditions.

**Phenolics extraction**
Phenolic compounds from two grams of ground samples were extracted with 20 ml of methanol using the Soxtec System HT (1043 – Tecator) for 1h 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 16,250×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 Na₂CO₃ (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 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 mg of gallic acid equivalent/g dry base of fermented soybeans (mg GAE/g 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

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

[1]

**Numerical and statistical methods**

Fitting procedures and parametric estimations calculated from the results were performed by minimisation of the sum of quadratic differences between observed and model/equation predicted values, using the non linear 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 formalised 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*) was characterised 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 reparameterised logistic equation shown below (23-27). As it can be observed, all the bioproductions monitoring were improved with the fermentation process. In Table 1 the notation and the dimensions of the parameters are summarized.

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

A similar reparameterised 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 \((Y_0)\).

\[
Y = \frac{Y_f}{1 + \exp \left[ 2 + \frac{4 \cdot v_{my} \cdot (\lambda_y - t)}{Y_f} \right] + Y_0} \quad [3]
\]

In the previous equation \(Y\) represents the variable fitted with equation 3 (total phenolic, DPPH scavenging and enzyme production). The limit of the function
as time approaches infinite (when the antioxidants production and the enzymatic activity are maxima) gives

\[ \lim_{t \to \infty} Y = Y_m = \frac{Y_i}{1 + \exp\left(-\frac{4 \cdot v_{my} \cdot \left(\lambda_y - \infty\right) + 4 \cdot Y_0}{Y_i}\right)} + Y_0 = Y_1 + Y_0 \]  

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

\( A. \) \emph{oryzae} led to a higher and faster growth in soybeans (\( N_m = 4.96 \) cells/g and \( \mu_{mn} = 2.29 \) d\(^{-1}\)). This strain also showed the 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\(^{-1}\)d\(^{-1}\) and \( v_{md} = 14.54 \) %/d) and the smaller antioxidant production lag phase (\( \lambda_d = 2.94 \) d).

In line with these results, the \( \beta \)-glucosidase activity of \( A. \) \emph{oryzae} and \( A. \) \emph{awamori} showed a similar pattern (see Figure 1). The \( A. \) \emph{oryzae} maximum enzymatic activity (\( E_m \)) was higher than \( A. \) \emph{awamori} activity; 42.12 U/mg and 39.62 U/mg respectively. However, the maximum rate of enzymatic production (\( v_{me} \)) and the lag phase (\( \lambda_e \)) of \( A. \) \emph{oryzae} were lower than those of \( A. \) \emph{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 $F$; $\alpha=0.05$), and the parametric estimations (with the exception of $\lambda_p$) were statistically significant (Student’s $t$; $\alpha=0.05$). All the predicted values obtained by the non-linear adjustments demonstrated high coefficients of linear correlation with the real observed values (see Table 2).

**DISCUSSION**

A number of analytic 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 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to accurate measuring 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). Based on 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 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 the antioxidant development during fermentation
has not been done before and comparisons with previous studies cannot be
done. During the last years, artificial neural networks (ANN) have been broadly
employed in the prediction of food processes (34-36). This mathematical tool,
based on biological neural networks, uses adaptive models that change its
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 in order to define
properties of the fermentations. The logistic equation proposed in our work has
a pseudo-mechanistic approach and have been widely used in the simulation,
prediction and control of fermentation processes (26, 37). The parameters
obtained from this model were biological and statistical significant and allowed a
comparison of the bioproductions from both *Aspergillus*.

Fermentation with *A. oryzae* achieved the maximum total phenolics
concentration of 25 mg of gallic acid/g dried soybeans after 5 days. A
maximum of 20 mg of gallic acid/g dried soybeans was obtained with A. *awamori* after 8 days. Lin et al. (8) found that the phenolics content of soybeans koji with *A. awamori* increased between 18 and 45.7 mg of gallic acid/g extract in 3 days, and up to 42 mg of gallic acid/g extract of the same koji with *A. oryzae*. Fernandez-Orozco et al. (38) also reported a phenolics concentration increment after 2 days 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) utilised different strains of *Aspergillus* to ferment soybeans, and though 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 process (8). Miura et al. (39) reported an increasing β-glucosidase activity in soybeans extract fermented with *Ganoderma lucidum* after 9 days, which was attributed to a decrease of isoflavone glycoside and the corresponding aglycones increment.
The 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 conclude 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.

**Acknowledgements**

The authors wish to acknowledge the SPMU-TPSDP Diponegoro University (Indonesia) for the grant awarded to Dyah Hesti Wardhani to do this research (ADB loan No. 1792-INO). This manuscript has been improved with the comments of three unknown referees.

**REFERENCES**


### TABLES CAPTIONS

**Table 1.** Notations used with units.

**Table 2.** Parametric estimations (see Table 1) corresponding to the kinetic models (2-4), applied to cultures of *A. oryzae* and *A. awamori* in soybeans. CI, confidence intervals (α=0.05); F, *F*-Fisher test (df₁: degrees of freedom for model; df₂: degrees of freedom for error); r, correlation coefficient between observed (obs) and predicted (pred) data; and NS, not significant.

### FIGURE CAPTION

**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 db). The error bars are the standard error of six measures (2 fermentation replicates × 3 independent analytical determinations).
## TABLE 1

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Dimensions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$</td>
<td>Cellular biomass</td>
<td>cells number/g db</td>
</tr>
<tr>
<td>$N_0$</td>
<td>Initial cellular biomass</td>
<td>cells number/g db</td>
</tr>
<tr>
<td>$t$</td>
<td>Time</td>
<td>days (d)</td>
</tr>
<tr>
<td>$K$</td>
<td>Maximum relative cellular biomass</td>
<td>Dimensionless</td>
</tr>
<tr>
<td>$\mu_{mn}$</td>
<td>Specific maximum growth rate</td>
<td>d$^{-1}$</td>
</tr>
<tr>
<td>$\lambda_0$</td>
<td>Growth lag phase</td>
<td>d</td>
</tr>
<tr>
<td>$N_m$</td>
<td>Maximum cellular biomass</td>
<td>cells number/g db</td>
</tr>
<tr>
<td>$E_m$</td>
<td>Maximum enzymatic activity</td>
<td>U/mg</td>
</tr>
<tr>
<td>$v_{me}$</td>
<td>Maximum enzymatic production rate</td>
<td>U.mg$^{-1}$.d$^{-1}$</td>
</tr>
<tr>
<td>$\lambda_e$</td>
<td>Enzymatic activity lag phase</td>
<td>d</td>
</tr>
<tr>
<td>$P_m$</td>
<td>Maximum total phenolics</td>
<td>mg of gallic acid equivalent/g dry base of soybeans (mg GAE/g db)</td>
</tr>
<tr>
<td>$v_{mp}$</td>
<td>Maximum total phenolics production rate</td>
<td>mg GAE.g$^{-1}$.d$^{-1}$</td>
</tr>
<tr>
<td>$\lambda_p$</td>
<td>Phenolics activity lag phase</td>
<td>d</td>
</tr>
<tr>
<td>$D_m$</td>
<td>Maximum DPPH scavenging activity</td>
<td>%</td>
</tr>
<tr>
<td>$v_{md}$</td>
<td>Maximum DPPH inhibition rate</td>
<td>%/d</td>
</tr>
<tr>
<td>$\lambda_d$</td>
<td>DPPH activity lag phase</td>
<td>d</td>
</tr>
</tbody>
</table>
### TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>Aspergillus oryzae</th>
<th>values ± CI</th>
<th>Aspergillus awamori</th>
<th>values ± CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROWTH (N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>4.963 ± 0.145</td>
<td></td>
<td>4.639 ± 0.614</td>
<td></td>
</tr>
<tr>
<td>K₄₉₉₉</td>
<td>2.292 ± 0.440</td>
<td></td>
<td>1.743 ± 0.811</td>
<td></td>
</tr>
<tr>
<td>λₙ</td>
<td>0.894 ± 0.234</td>
<td></td>
<td>3.234 ± 0.681</td>
<td></td>
</tr>
<tr>
<td>Nₙ</td>
<td>(1.66 ± 0.05) x 10³</td>
<td></td>
<td>(1.39 ± 0.18) x 10³</td>
<td></td>
</tr>
<tr>
<td>F (df₁=3; df₂=6; α=0.05)</td>
<td>3085.30</td>
<td></td>
<td>238.06</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>r (obs-pred)</td>
<td>0.9987</td>
<td></td>
<td>0.9927</td>
<td></td>
</tr>
<tr>
<td>β-GLUCOSIDASE ACTIVITY (E)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eₙ</td>
<td>42.117 ± 2.504</td>
<td></td>
<td>39.620 ± 2.590</td>
<td></td>
</tr>
<tr>
<td>Vₑ</td>
<td>23.537 ± 12.551</td>
<td></td>
<td>27.451 ± 19.323</td>
<td></td>
</tr>
<tr>
<td>F (df₁=4; df₂=5; α=0.05)</td>
<td>581.82</td>
<td></td>
<td>469.48</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>r (obs-pred)</td>
<td>0.9945</td>
<td></td>
<td>0.9959</td>
<td></td>
</tr>
<tr>
<td>TOTAL PHENOLICS (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pₙ</td>
<td>24.149 ± 2.848</td>
<td></td>
<td>21.010 ± 4.777</td>
<td></td>
</tr>
<tr>
<td>Vₚₚₚ</td>
<td>5.580 ± 4.699</td>
<td></td>
<td>3.183 ± 1.868</td>
<td></td>
</tr>
<tr>
<td>F (df₁=4; df₂=5; α=0.05)</td>
<td>239.68</td>
<td></td>
<td>317.08</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>r (obs-pred)</td>
<td>0.9775</td>
<td></td>
<td>0.9859</td>
<td></td>
</tr>
<tr>
<td>DPPH SCAVENGING (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dₙ</td>
<td>53.980 ± 3.275</td>
<td></td>
<td>50.659 ± 6.288</td>
<td></td>
</tr>
<tr>
<td>Vₙₚₚₚ</td>
<td>14.542 ± 7.160</td>
<td></td>
<td>11.206 ± 7.130</td>
<td></td>
</tr>
<tr>
<td>F (df₁=4; df₂=5; α=0.05)</td>
<td>811.99</td>
<td></td>
<td>315.52</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>r (obs-pred)</td>
<td>0.9938</td>
<td></td>
<td>0.9878</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 1