

Full Length Research Paper

Kinetic models of cell growth, substrate utilization and bio-decolorization of distillery wastewater by *Aspergillus fumigatus* U_{B2}60

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Bio-decolorization kinetic studies of distillery effluent in a batch culture were conducted using *Aspergillus fumigatus*. A simple model was proposed using the Logistic Equation for the growth, Leudeking-Piret kinetics for bio-decolorization, and also for substrate utilization. The proposed models appeared to provide a suitable description for each parameter devoted to the growth phase. The biomass yield for 14 g/l substrate was 70.7%. The maximum specific growth rate (μ_m) of the obtained and fitted data is close to the calculated μ_m of the present research work (0.03 h^{-1}). It was found that the kinetic model for the bio-decolorization of distillery effluent was growth associated.

Key words: Bio-decolorization, kinetic models, distillery effluent, Logistic Equation, leudeking-Piret kinetics.

INTRODUCTION

World demand for ethanol from carbohydrates via fermentation process is very attractive, because of the depletion of fossil fuel, limited non-renewable energy resources and fluctuation of oil and natural gas prices. Among the carbohydrates used for ethanol production, molasses are the most available raw material, with low costs and also involved in less sophisticated fermentation

process, by which ethanol is easily produced. The effluent from molasses is based on distilleries that contain large amounts of dark brown colored substances (melanoidin) which may create many problems and also environmental pollution (Angayarkanni et al., 2003; Beltran et al., 2001; Najafpour and Cheong, 2003).

Ethanol production from distilleries usually varies from 5 to 12% (by volume) hence a large volume of effluent is produced. This can be accounted for 85 to 95% of the total volume. Therefore, on averaged base of molasses utilized in distillery produces 10-15 liters of effluent (spent wash) per liter of alcohol produced (Jimenez et al., 2003).

The high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of the distillery effluents typically ranged between 35000 - 50000 and 100000 - 150000 mg/l, respectively (Nandy et al., 2002; Shayegan et al., 2005).

Recently, an increasing attention is being directed toward utilization of microbial activities (pure bacteria and fungi) for the decolorization and mineralization of distillery effluent (Pant and Adholeya, 2007; Pazouki et al., 2006). Microbial species such as *Bacillus megaterium*, *Bacillus cereus* (Jain et al., 2002), *Pseudomonas fluorescens*

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Abbreviations: m_s , Maintenance coefficient (g substrate/g cells); P, biodecolorization responsible enzyme (metabolite) (g/l); S, substrate concentration (g/l); S_0 , initial substrate concentration (g/l); T, biodecolorization time (h); X, cell concentration (g/l); X_m , maximum cell concentration (g/l); $Y_{X/S}$, yield factor for cells on carbon substrate (g cells/ g substrate); α , growth-associated product formation coefficient (g/g); β , non-growth-associated product formation coefficient (g/g.h); and μ_m , maximum specific growth rate (h^{-1}).

(Dahiya et al., 2001a), *Trametes versicolor* (Benito et al., 1997), *Geotrichum candidum* (Kim and Shoda, 1999), *Coriolus hirsutus* (Miyata et al., 2000), *Aspergillus fumigatus* UB2 60 (Pazouki et al., 2006), *Mycelia sterilia* (Sirianuntapiboon et al., 1988), *Flavodon flavus* (Raghukumar et al., 2004), *Rhizoctonia* sp. D-90 (Sirianuntapiboon et al., 1995), *Coriolus versicolor* (Ohmomo et al., 1985) have been employed for biodecolorization of distillery effluent. The present research work is unique and promising; there are numbers of reports and literature related to this research field, but the problem yet exist to adopt these bench cases to large scale distillery effluent treatment process. The mechanism of biodecolorization of distillery effluent by the removal of melanoidin is not completely understood but there are some evidences of involvement of lignolytic enzymatic mechanisms (Benito et al., 1997). Enzymes such as sugar-oxidase (Watanabe et al., 1982), peroxidases and extracellular H₂O₂ produced by glucose-oxidase (Miyata et al., 2000) are studied for their active degradation of melaroidins. The mechanism of biodecolorization of melonoidins by *A. fumigatus* is yet to be studied.

In the previous studies, the procedures of microorganisms screening was reported (Pazouki et al., 2006). *A. fumigatus* UB2 was employed in a continuous reactor (Shayegan et al., 2005) UV irradiated the spores of *A. fumigatus* UB2 and optimized the important variables to decolorize distillery effluent (Pazouki et al., 2006). In the present study, an unstructured model was used to describe the relationship between principle states of variables and explain quantitatively the behavior of biodecolorization system. The model can provide insights to the analysis conducted during the course of operation of the fermenter. In this study, the unstructured model was used since these types of models are much easier to apply and they are accurately proved that the model can express many fermentation processes. Yet, to our knowledge, no investigations have been carried out on unstructured model for biodecolorization of distillery effluent.

MATERIALS AND METHODS

Microorganism

The fungus used was originally isolated from soil sample taken at Bidestan Distillery and Food Products, Qazvin, Iran, identified as *A. fumigatus*, strain UB2 60, and maintained on potato dextrose agar (Pazouki et al., 2006).

Inoculum development

An aqueous suspension of spores from a 7-day agar slant was prepared. The spores enumerated by haemocytometer and aliquots containing 8×10^6 spores were transferred into each of a series of 500 ml Erlenmeyer flasks containing 100 ml, 7% glucose-mineral salt medium (Shayegan et al., 2005). After incubation on a rotary shaker (160 rpm) at 30°C for 3 days, the resultant mycelial pellets

were then separated from the medium by centrifugation under aseptic conditions at 4000 g for 10 min, and different amounts used for decolorization of MSW as described below.

Decolorization experiments

For the decolorization experiments, wastewater sample was obtained from Bidestan Distillery and Food Products, Qazvin, Iran, where it had been treated in an up-flow aerobic sludge blanket reactors with working volume of 200 m³. The fungus was grown at 30°C in 100 ml medium (previously autoclaved at 121°C for 15 min) in a series of 500 ml Erlenmeyer flasks on a rotary shaker operating at 160 rpm. One liter of the distilled wastewater sample consists of 15.3 g maltose, 0.75 g NaNO₃, 0.15 g KH₂PO₄, 4 ml glycerol and the sample was inoculated with 2.75 g fungal biomass. The initial pH of the medium in the shaking flask was 5.57.

Assays

Defined volume of samples (10 ml) were drawn every 12 h from the shaking flasks, centrifuged at 4000 g for 10 min and the supernatants used for determination of decolorization efficiency (Dahiya et al., 2001b; Fitzgibbon et al., 1995; Nelson, 1994; Somogyi, 1952) and maltose concentration (Elibol and Mavituna, 1999; Patil et al., 2003). The precipitant was used to calculate the mycelial mass. Decolorization experiments were performed in duplicate culture and analysis were carried out in duplicate. The presented data are the averaged value of the measurements. The decolorization in each sample was measured as absorbance at wavelength of 475 nm using a Pye Unicam spectrophotometer and was expressed as a percentage of the original absorbance prior to biological treatment.

Kinetic model

The simplest kinetic model expresses the stoichiometric relation for product formation and substrate utilization is unstructured model. The unstructured model was used. The rate equation is expressed by the biomass concentration (*X*), decolorized product (*P*) and maltose as substrate concentration (*S*) to describe the decolorization process.

Microbial growth

Under optimal growth condition and when the inhibitory effects of substrate and product were neglected, the rate of cell growth follows the well-known exponential relation. The simplest relationship describes is unstructured Malthus model (Najafpour, 2007; Najafpour and Yap, 2005; Zinatizadeh et al., 2006):

$$\frac{dX}{dt} = \mu X \quad (1)$$

Where, the constant μ is defined as the specific growth rate. Equation 1, thus implies that *X* increases with respect to time regardless of substrate availability. In reality, the growth of all is governed by hyperbolic relationship and there is a limit to the maximum attainable biomass concentration. It was assumed that the limiting substrate is consumed according to first-order reaction kinetics:

$$-\frac{dS}{dt} = k_s S$$

Where k_S is rate constant. The yield of biomass ($Y_{x/s}$) is based on utilized substrate which is defined as follows:

$$Y_{x/s} = -\frac{\Delta X}{\Delta S} = -\frac{X - X_0}{S - S_0} \quad (2)$$

Where X_0 is inoculum's concentration and S_0 is initial substrate concentration in g/l, respectively. Rearranging equation 2 gives:

$$S = \frac{X_0 + Y_{x/s}S_0 - X}{Y_{x/s}} \quad (3)$$

Maximum cell dry weight is equal to sum of the inoculum's size and the coefficient yield multiplied by substrate concentration in the inoculum, with the assumption of substrate is converted to biomass. Inserting

$X_m = X_0 + Y_{x/s}S_0$ into equation 3 yields as follows:

$$S = \frac{X_m - X}{Y_{x/s}} = \frac{X_m}{Y_{x/s}} \left(1 - \frac{X}{X_m}\right) \quad (4)$$

Applying the change of substrate with respect to time and the chain rule principle on the right-hand side of the above rate equation:

$$\frac{dS}{dt} = \frac{dS}{dX} \cdot \frac{dX}{dt}, \text{ the rate equation for the formation of biomass is:}$$

$\frac{dX}{dt} = k_S Y_{x/s} S$ then, equation 4 is incorporated into the rate equation gives:

$$\frac{dX}{dt} = k_S X_m \left(1 - \frac{X}{X_m}\right) \quad (5)$$

Equation 5 contributes to the postulated model which is induced by an inhibition factor for the population growth rate. Assuming that the inhibition is second-order with respect to cell dry weight (X^2), then the equation becomes:

$$\frac{dX}{dt} = \mu_m \left[1 - \frac{X}{X_m}\right] X \quad (6)$$

Where X_m is the maximum biomass concentration in $g.l^{-1}$ and μ_m is maximum specific growth rate in h^{-1} . This equation is known as the Riccati equation (Najafpour, 2007), using the boundary condition at $t=0$ then $X = X_0$, gives a sigmoidal variation of X as a function of time. Equation 6 can be easily integrated to give the logistic equation which may represent both an exponential and a stationary phase. The resulted equation is shown in equation 7:

$$X = \frac{X_0 X_m e^{\mu_m t}}{X_m - X_0 + X_0 e^{\mu_m t}} = \frac{X_0 e^{\mu_m t}}{1 - (X_0 / X_m)(1 - e^{\mu_m t})} \quad (7)$$

The batch culture is a closed system, which would only maintain cell viability for a limited time, and the growth cycle changes progressively from one phase to another in the remaining media and environment conditions. The logistic equation presented above,

does not predict the death phase of microorganisms after the stationary phase. To predict the death phase of bacteria after the stationary phase, is expressed in equation 8 (Najafpour, 2007).

$$X = \frac{X_0 e^{\mu_m t}}{1 - \left(\frac{X_0}{X_m}\right)^2 \left(\frac{\mu_m}{k + \mu_m}\right) \left[1 - e^{(k + \mu_m)t}\right]} \quad (8)$$

Where, k is a constant value, which is associated with the promotion or decline of cell population in the batch culture. On the other hand, the positive value of k shows the promotion of the cell population whereas a negative value of k shows a decline in the cell population.

Decolorization percent

The kinetic study of Leudeking and Piret on lactic acid fermentation using *Lactobacillus delbruekii* indicated that the kinetics of product formation is a combined rate known as growth associated and non-growth associated (Bailey and Ollis, 1986). Therefore product

formation rate, $\frac{dP}{dt}$ allows a correlation between the cell mass and

product concentration. In this study, the decolorization was assumed as growth related model, that is due to the presence of an enzyme and hence the model is related to the product formation. The rate of product formation is expressed by Leudeking and Piret rate model as follows:

$$\frac{dP}{dt} = \alpha X + \beta \frac{dX}{dt} \quad (9)$$

This two-parameter kinetic expression was proven extremely useful and versatile in fitting product formation data for many different fermentation processes which is known as Leudeking and Piret kinetic expression. For the growth associated product formation (i.e. when $\alpha = 0, \beta \neq 0$) equation 9 can be simplified as follows:

$$P = K + \beta X \quad (10)$$

Substrate uptake

A carbon substrate such as maltose is used to support cell viability, even in the absence of growth. These activities include cell motility, enzyme turnover, osmotic work, nutrient storage and other processes referred to as maintenance function. The substrate consumption equation used is similar to Leudeking-Piret kinetic equation in which the amount of carbon substrate used for the product formation is assumed to be negligible.

$$\frac{dS}{dt} = m_s X + \frac{1}{Y_{x/s}} \frac{dX}{dt} \quad (11)$$

Substituting equation 7 into Equation 11 then performing integration, the following equation is yielded:

$$S = S_0 - \frac{X_0 e^{\mu_m t}}{Y_{x/s} [1 - (X_0 / X_m)(1 - e^{\mu_m t})]} + \frac{X_0}{Y_{x/s}} - \frac{X_m m_s}{\mu_m} \ln[1 - (X_0 / X_m)(1 - e^{\mu_m t})] \quad (12)$$

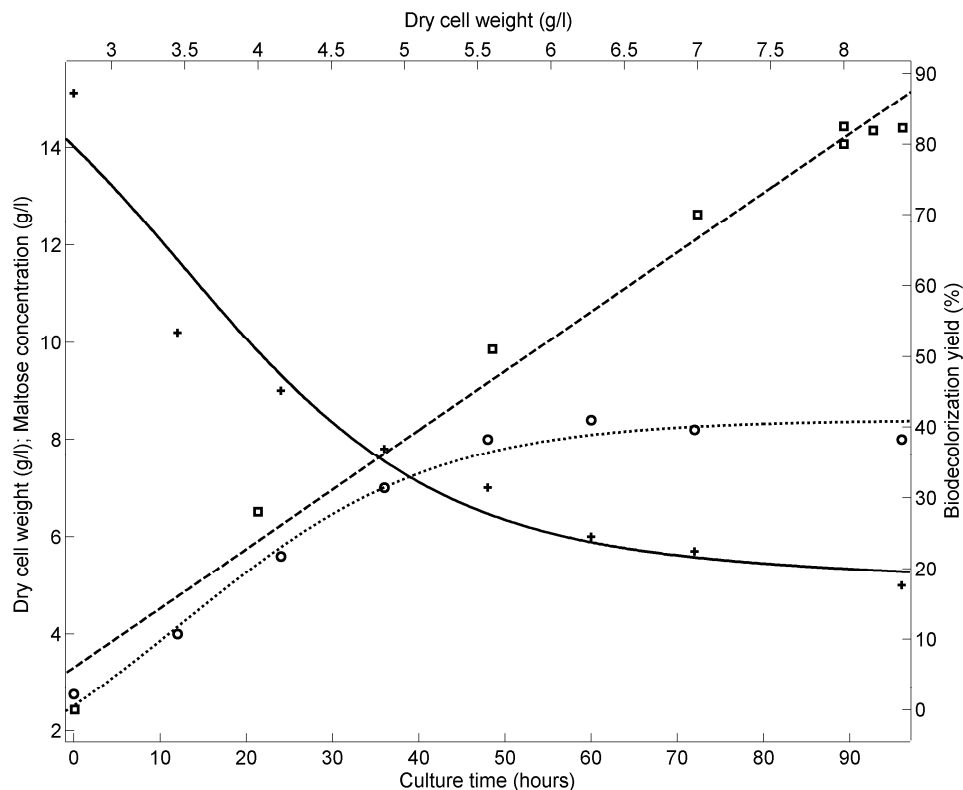


Figure 1. Comparison of experimental data and calculated values for the proposed models. (○) experimental, and (...) calculated value of dry cell weight concentration vs. time, (□) experimental, (---) calculated value of biodecolorization vs. dry cell weight concentration, (+) experimental, and (—) calculated value of maltose concentration vs. time.

In this study, equations 7, 8, 10, and 12 were used to simulate the experimental results. The Matlab software was employed to estimate the values of constant parameters, and the curve fitting was based on the proposed model.

RESULTS AND DISCUSSION

Fungus growth kinetics

The lag phase of *A. fumigatus* in decolorization cultivation was very short as the fungus was already adapted before it was used for decolorization. Therefore the cells entered the exponential phase instantly. The strain started to decolorize the effluent when the cells entered the exponential phase. Therefore decolorization and cells growth took place simultaneously. Maximum cell concentration in the experimental data was 8.4 g/l ($X_m = 8.4$ g/l). By fitting the experimental data into equation 7, the parameters of equation obtained were $\mu_m = 0.0685 \text{ h}^{-1}$ and $X_0 = 2.52$ g/l. Figure 1 shows the experimental data of dry cell weight versus time (circle markers), and the model fitted into equation 7 based on these data (dot line). This figure, also presents maltose utilization along with biomass generation and biodecolorization. Coefficients of determi-

nation (R-square), and Adjusted R-square of the model were 0.987, and 0.985, respectively and indicate that the data are perfectly fitted the rate model. Also, according to the fitted growth model, the calculated values of X_0 (2.5 g/l) were quite satisfactory with the experimental value (2.75 g/l). The lower value of calculated cell concentration compared to the experimental data was attributed to the viability of cells. Less than 100% viability may yield on X_0 value less than the measured initial cell concentration (Elibol and Mavituna, 1999; Najafpour, 2007). The data points for substrate are sharply reducing while the biomass concentration is exponentially produced.

In order to describe the cell population with inhibition or promotion, the experimental data were fitted into equation 8. Results are depicted in Figure 2 (solid line). The coefficients for the equation calculated are $\mu_m = 0.03 \text{ h}^{-1}$, $X_0 = 3.1$ g/l, and $k = 0.014$. Determination coefficient and adjusted R-square are 0.98, and 0.973, respectively. The maximum cell dry weight concentration (X_m) was 5.1 g/l, when the inhibition value was 0.02 h^{-1} . The maximum cell dry weight reached 16.84 g/l when the growth inhibition ($k=0$) was not observed.

For further investigation on fitting the experimental

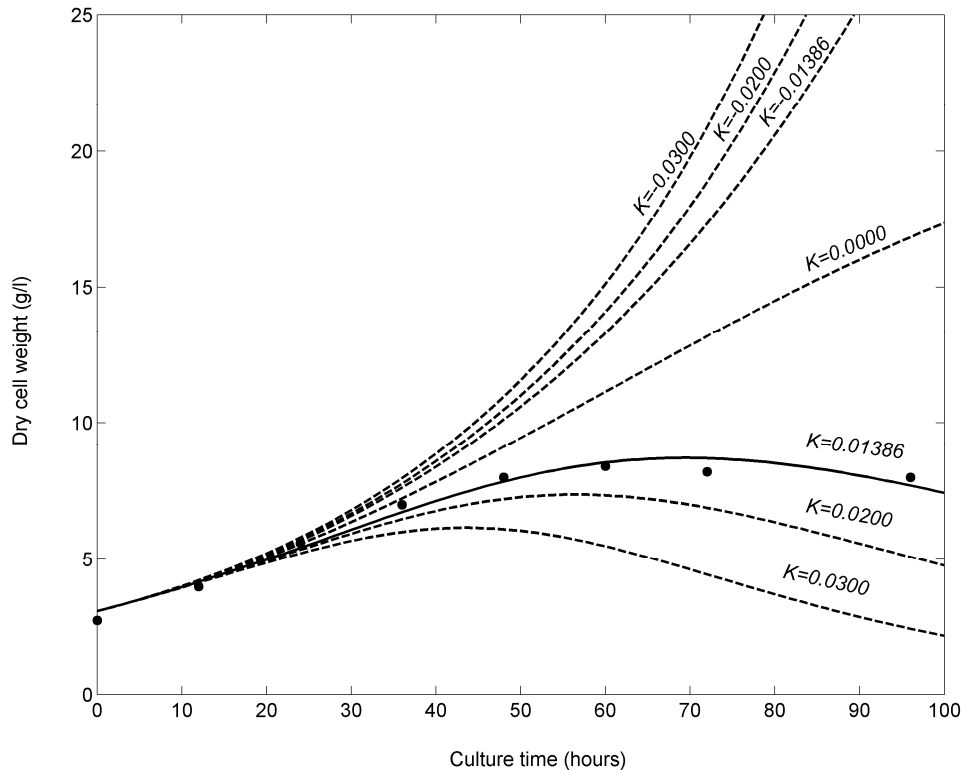


Figure 2. Growth simulation of *Aspergillus fumigatus* UB2, considering the inhibition/promotion value (K). (●) experimental, and (—) calculated value, (---) calculated value reported in the literature for K .

data of decolorization process of molasses wastewater, and to make a comparison with another similar work, the experimental data reported by Ohmomo et al. (1985), using *A. fumigatus* G-2-6 have been fitted using suitable kinetic models described, and have been shown in Figure 3. For the cell growth kinetic, the kinetic parameters μ_m and X_0 are 0.0644 h^{-1} , and 1.59 g/l , respectively; also, calculated initial cell concentration (X_0) was close to the experimental value (2.0 g/l). R-square (0.997) and Adjusted R-square (0.996) imply the model has perfectly matched the experimental data. It can be seen that the maximum specific growth rate (μ_m) obtained by Ohmomo et al. model is similar to the value obtained in the present work.

Furthermore, matching the experimental data reported by Ohmomo et al. into equation 8, the calculated values are $\mu_m = 0.032 \text{ h}^{-1}$, $X_0 = 2.54 \text{ g/l}$, and $k = 0.02$. Determination coefficient and adjusted R-square are 0.988, and 0.976, respectively. The maximum specific growth rate (μ_m) of the obtained and fitted data is close to the calculated μ_m of the present research work (0.03 h^{-1}), but the calculated inhibition value (k) is higher than that of this work (0.014). The maximum cell dry weight concentration (X_m) was 7.39 g/l , when the inhibition value was 0.03 h^{-1} . The maximum cell dry weight reached 32.9 g/l

when the growth inhibition ($k=0$) was not observed (Figure 4).

Biodecolorization

The experimental data for biodecolorization was fitted into equation 10. The values obtained by Matlab were $K = -33.1$ and $\beta = 14.25$. Therefore, the relationship between biodecolorization and dry cell weight concentration is represented to be $P = -33.1 + 14.25X$ with correlation coefficient (R-square) = 0.984, and adjusted R-square = 0.981. The above relationship indicates that biodecolorization is absolutely linear (Figure 1), which is strongly related to the cell growth. It is evident that biodecolorization can be attributed to a growth associated type. In the model, β (14.25 g/g) is the growth associated biodecolorization coefficient and may be identified with the product on biomass yield $Y_{X/P}$.

Fitting the data reported by Ohmomo et al. into equation 10, it can be concluded that the polynomial model was matched and very well fitted with the experimental data with the R-squared (0.90) and adjusted R-square (0.87) values which were near to 1. Also, the

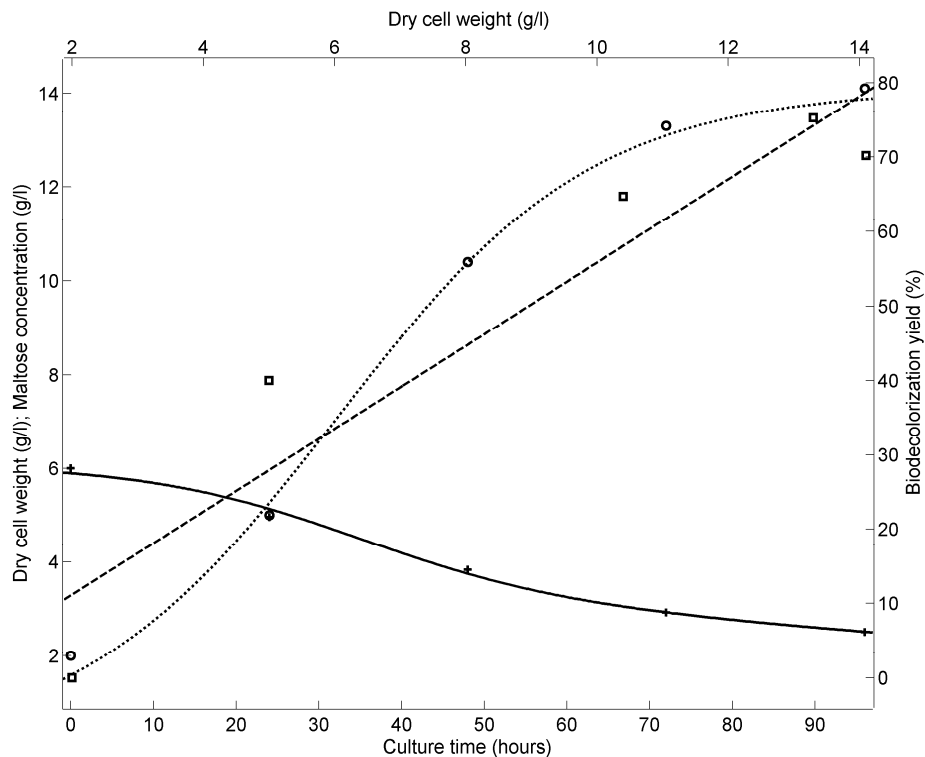


Figure 3. Comparison of experimental data reported by Ohmomo et al., and calculated values for the proposed models. (○) experimental, and (...) calculated value of dry cell weight concentration vs. time, (□) experimental, (---) calculated value of biodecolorization vs. dry cell weight concentration, (+) experimental, and (—) calculated value of maltose concentration vs. time.

model constants, K and β , were calculated as 0.00334, and 5.584, respectively. Figure 3 shows the decolorization kinetic model with a dashed line.

Substrate uptake

Experimental data were fitted into equation (12). The values of maltose uptake model, obtained by Matlab software, were $Y_{x/s} = 0.707 \text{ g/g}$, $S_0 = 14.03 \text{ g/l}$, and $m_s = 0.0007 \text{ g/g}$. The fitted experimental data for the biomass growth curve is shown in Figure 1. The data points have followed exactly the same pattern as the projected model. R-square, and adjusted R-square are 0.95, and 0.926, respectively. So the results of curve fitting for the experimental data and equation 12 were quite satisfactory.

Moreover, fitting the experimental data reported by Ohmomo et al. into equation 12, the equation coefficients calculated. $Y_{x/s} = 5.052 \text{ g/g}$, $S_0 = 5.89 \text{ g/l}$, and $m_s =$

0.00093 g/g . Also, R-square, and adjusted R-square are 0.994, and 0.988, respectively. Therefore, it can be concluded that the model presented in Figure 3 by a solid line, is very well fitted with the experimental data.

Testing the model

The obtained model was examined for the microbial growth, biodecolorization and maltose uptake. Also, the obtained data for several batch experiments were compared. The calculated values using the growth related parameters were evaluated for several batches of biodecolorization. The error analysis for all of the calculated values was less than 10%. It was found that these models can be used to describe the biodecolorization of distillery effluent by *A. fumigatus* (Bailey and Ollis, 1986; Najafpour, 2007; Najafpour and Yap, 2005). Figure 1 shows about 70% of total carbohydrate was utilized for the purpose of biodecolorization, while the pigments of melanoidin caused the darkness of effluents were removed. More than 80% of the pigments from Bidestan Distillery's effluent were removed via biological activities of *A. fumigatus*.

Conclusion

Biodecolorization of distillery effluent is a very complex process, and it is often very difficult to obtain a perfect picture of what is actually occurred in the biological processes. The model presented in this work is able to fit

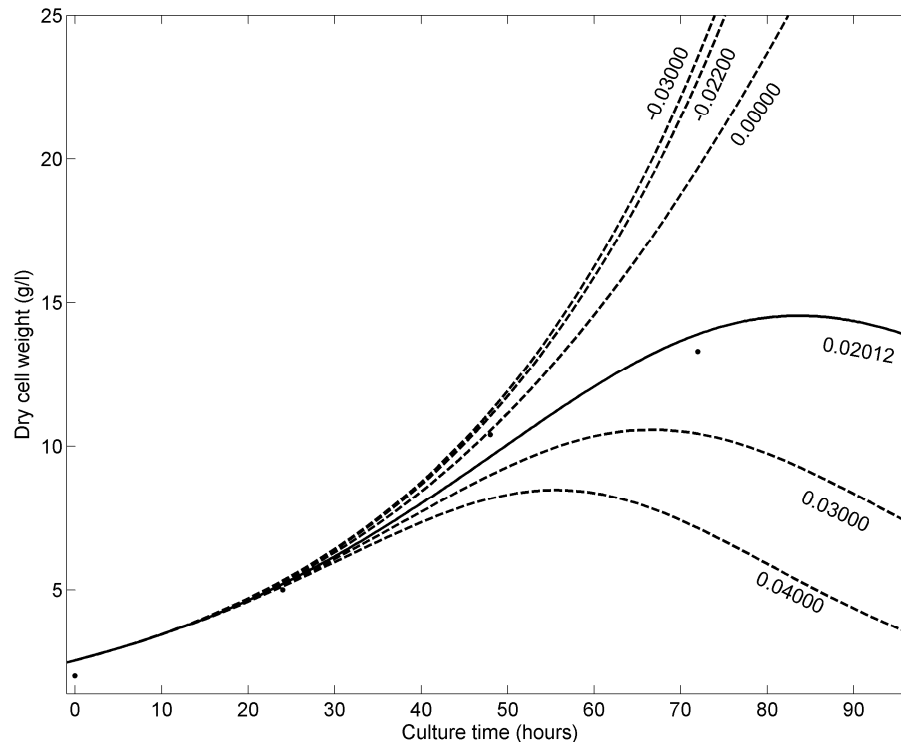


Figure 4. Growth simulation of *Aspergillus fumigatus* G-2-6 (Ohmomo et al., 1995), considering the inhibition/promotion value (K). (●) experimental, and (—) calculated value, (---) calculated value reported in the literature for K .

not only the experimental data given in this work but also data obtained from the literature. Maximum biomass concentration (X_m) is one of the important limitation parameter of the model. This means prior to any analysis, X_m has to be defined experimentally. Therefore, in order to predict the values to model, an actual value of X_m is required.

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