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Effects of Tetrazolium Chloride Concentration, O_2, and Cell Age on Dehydrogenase Activity of *Aspergillus niger*

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Abstract

The effects of triphenyl tetrazolium chloride (TTC) concentration, cell age, and presence of O_2 on the dehydrogenase activity of *Aspergillus niger* as measured by triphenyl formazan (TF) yield were investigated. The results indicated that increasing TTC concentration initially increased the TF yield and then decreased it. The maximum TF yield was observed at a TTC concentration of 30 g/L for young cells (4 d old) and 20 g/L for old cells (12 d old). Conducting the test under anaerobic conditions increased the TF yield. About 18% of the TF produced was converted back into TTC in the presence of oxygen. The relationship between dehydrogenase activity of *A. niger* (as measured by TF yield) and cell mass was found to be linear. A kinetic model describing the relationship between reaction rate (micromoles of TF formed per hour) and TTC concentration while accounting for substrate inhibition was developed, and the model constants were calculated. The optimum TTC-test conditions for dehydrogenase activity measurement of A. niger were a TTC concentration of 20 g/L, a pH of 9.0, a temperature of 55°C, an incubation time of 3 h, and anaerobic conditions.

Index Entries: Dehydrogenase activity; *Aspergillus niger*; triphenyl tetrazolium chloride; triphenyl formazan; cell age.

Introduction

Aspergillus niger is a filamentous fungi that is widely employed in industrial biotechnology. It is capable of producing organic acids (citric and gluconic acids) and several enzymes that can be used in different industrial applications *(1–3)*. Pandey *(4)* reported that *A. niger* can produce more

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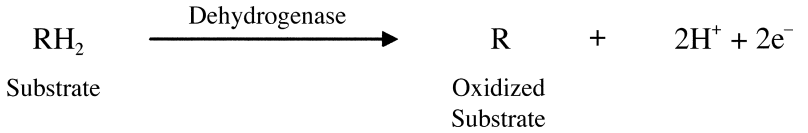
than 19 types of enzymes: α-amylase, β-amylase, maltase, lactase, catalase, proteinase, trehalase, tannase, dipetalase, polypetalase, lipase, cellulase, amidase, glucose oxidase, glucose dihydrogenase, urease, insulase, melibase, and zymase. A. niger species also contains chitin in its cell wall (42.0% dry weight basis), which is a versatile environmentally friendly biopolymer *(5,6)*. Therefore, in these biotechnological applications, understanding the metabolic activity of *A. niger* is very important.

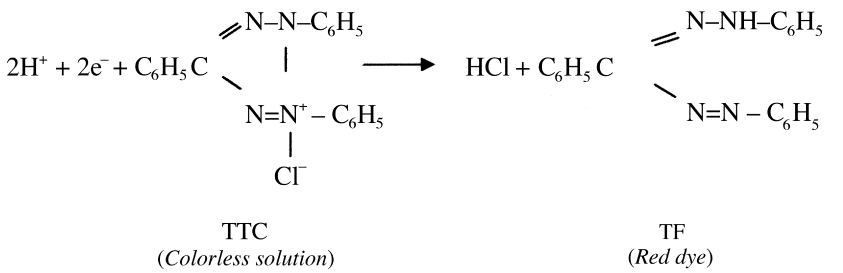
One potential method of readily monitoring the metabolic activity of *A. niger* is through the measurement of dehydrogenase activity. Dehydrogenase activity has been used for measuring microbial metabolic activity in soils *(7–17)*, sludges *(18)*, and lake sediments *(19)*, and for assessing fungal spore viability *(20–26)*.

Dehydrogenase activity also has been used for measuring the microbial growth in bioreactors using redox-sensitive dyes such as triphenyl tetrazolium chloride (TTC). TTC has been used for measuring microbial population in swine manure (27), single-cell protein (28), and ethanol (29). Measuring dehydrogenase activity using TTC involves incubation of the sample in which the dehydrogenase activity is to be measured with TTC in the presence of electron-donating substrate. The mechanism can be described by the following equations (18):

(a) Biologic oxidation of organic compounds:



(b) Chemical reduction of tetrazolium salts:



Coupling the oxidation of the substrate with the reduction of colorless TTC gives a highly colored triphenyl formazan (TF) that can be extracted by a solvent, and the color intensity can be measured colorimetrically using a spectrophotometer at the maximum spectra of the formazan solvent system. Generally, one unit of dehydrogenase activity is the amount of enzyme that would reduce 1 μmol of tetrazolium salt to 1 μmol of its formazan/min *(30)*. Since measurement of dehydrogenase activity using TTC depends on enzymatic activity, modification of the reaction environmental parameters should lead to significant improvement of the assay *(26)*. In our work we *(31)* noted that medium pH, incubation temperature,

and incubation period are critical parameters that affect the accuracy of dehydrogenase activity measurements. However, the enzymatic activities are significantly different for different microorganisms and may be affected by cell age *(26)* and the presence of oxygen *(32)*. Several investigators *(33–35)* also indicated that a high concentration of TTC may inhibit the growth and viability of microorganisms. It is therefore necessary to investigate the sensitivity of the test to TTC concentration, presence of O_2, and cell age in order to produce credible results when measuring microbial activity.

The objectives of the present study were (1) to investigate the applicability of the TTC test for quantifying the growth of the fungal species *A. niger*; and (2) to quantify the effects of TTC concentration, presence of O_2, and cell age on biochemical reduction of TTC to TF by *A. niger*.

Materials and Methods

Microorganisms

A. niger (ATCC 6275) was obtained from the American Type Culture Collection (Rockville, MD) as freeze-dried cells. The freeze-dried cells were revived in 6 mL of sterilized 0.1% (w/v) peptone solution prepared by dissolving 1 g of bacto-peptone (Difco, Detroit, MI) in 1 L of deionized distilled water and then sterilizing in an autoclave (Model No. STM-E; Market Forge Sterilmatic, NY) at 121°C and 103.4 kPa for 30 min. The microbial culture was kept in the peptone solution for 24 h at room temperature (24ºC). The rehydrated *A. niger* was transferred to potato dextrose broth (PDB), which contained infusion from 200 g of potatoes (4 g/L) and 20 g/L of bacto dextrose. A spore stock suspension was obtained by growing the fungus at room temperature (24ºC) for 4 d on potato dextrose agar, which contained infusion from 200 g of potatoes (4 g/L), 20 g/L of bacto dextrose, and 15 g/L of bacto agar. The conidia were harvested from the surface by adding sterilized deionized distilled water containing 0.01% (v/v) Tween-80 and scraping the surface with a sterilized spatula. The spore concentration was obtained using a direct standard plate count method *(36)*. The prepared suspension was then stored in a refrigerator at 4ºC until needed.

Reagents

The chemicals used in performing the dehydrogenase activity tests included 0.05 M Tris ([hydroxymethyl] aminomethane [$NH_2C(CH_2OH)_3$]) buffer solution (pH ~ 8.0), glucose, and 2:3:5 TTC salt. The Tris buffer was used to control the pH of the samples. The 0.05 *M* buffer was prepared by dissolving 6.057 g of Tris in 20 mL of 1.0 *N* HCl and bringing the solution to 1.0 L with distilled deionized water. The glucose served as an immediately bioavailable carbon supply, which is needed to enhance the reduction of TTC by microorganisms within the incubation period *(9)*. TTC was used as a colorimetric indicator. The TTC glucose solution was prepared by adding either 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, or

3.00 g of TTC ($C_{19}H_{15}N_4Cl$), depending on the required concentration, and 1 g of glucose ($CH_2OH[CHOH]_4CHO$) to a 100-mL volumetric flask and bringing the solution to 100 mL with distilled deionized water. The TTC solution is sensitive to light and was, therefore kept in the dark at 4°C.

Procedure

TTC Standard Curve

To determine the TF production from the reduction of TTC, a standard curve was developed from solutions of TF and ethyl alcohol with different concentrations. First, a standard solution of 0.0002 M TF was prepared by dissolving 0.03 g of TF ($C_6H_5N{:}NC[C_6H_5]{:}NNHC_6H_5$) in 500 mL of ethyl alcohol. Second, a set of nine solutions with TF concentrations of 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 _mol/50 mL were prepared. Finally, the absorbance of the prepared solutions was measured using a spectrophotometer (Spectronic 601; Fisher, Montreal, Quebec, Canada) at a wavelength of 484 nm and plotted against the known TF concentrations (mol/mL) as shown in Fig. 1. A blank sample was used to zero the spectrophotometer. The following linear relationship between the TF and the absorbance was obtained ($R^2 = 0.99$):

$$TF = 0.0603\ \bar{A}_{484} \tag{3}$$

in which TF is the TF concentration (μmol/mL) and $\bar{A}_{484}$ is the absorbance measured at 484 nm.

Preparation of *A. niger* Sample

A. niger cells were grown in shake flasks. One hundred fifty milliliters of PDB was added to each conical flask (250 mL). The flasks were plugged with sponge plugs, covered with tin foil, and autoclaved (Model No. STM-E; Market Forge Sterilmatic) at 121°C and 103.4 kPa for 30 min. After cooling, 1 mL of solution containing spores (70×10^6 colony-forming units/mL) was added into each flask, and the flasks were incubated for 4 d at room temperature (~24°C) in an incubator shaker (Series 25 Incubator Shaker; NB Scientific, Edison, NJ) running at 250 rpm. The organism grew as beads, which were harvested and blended in a blender to obtain a suspension of the fungus. Samples were then prepared by allowing 0.1 mL of *A. niger* vegetative cells suspension to grow at room temperature (~24°C) in 150 mL of PDB in the incubator shaker (model for either 4 or 12 d, during which the fungal cells formed clumps, which were then harvested and placed between two filter papers in order to remove the excess liquid before use in the tests. The moisture content of the fungal clumps was determined and the results of the TF yield were corrected and presented on a dry matter basis.

Determination of *A. niger* Dehydrogenase Activity

Each sample was transferred to a 50-mL centrifuge tube with a screw cap. Tris buffer solution (2.5 mL) was added to each tube, and the pH was

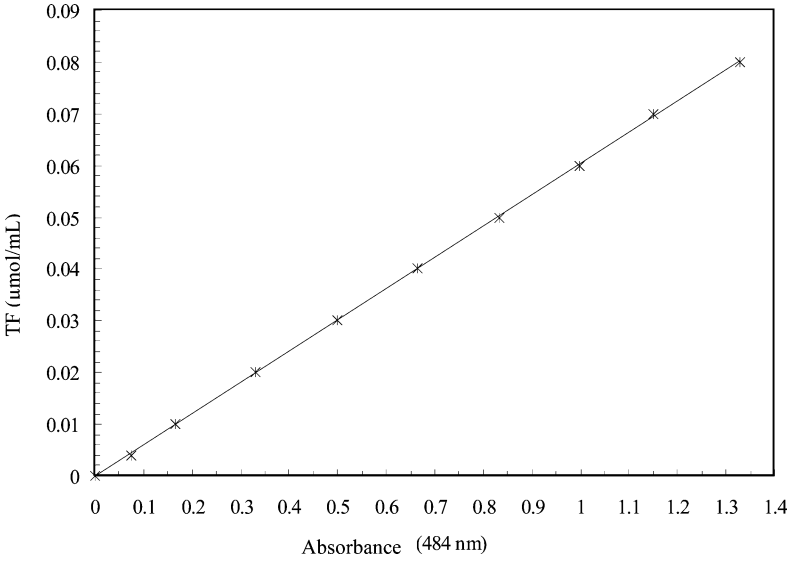


Fig. 1. TF standard curve.

adjusted to 9.0 using 1 *N* NaOH. For the control tubes, an aliquot of 0.5 mL of sterilized distilled deionized water was transferred to each tube instead of the TTC/glucose solution. For the tests that were conducted aerobically, a 0.5-mL aliquot of TTC/glucose solution with the desired concentration was transferred directly to each tube and the tubes were then tightly capped. For the tests that were conducted anaerobically, the contents of the tubes were flushed with nitrogen gas for 5 min, a 0.5-mL aliquot of TTC/glucose solution with the desired concentration was transferred to each tube while the nitrogen gas was still flushing the contents of the tubes, and the tubes were then tightly capped. A special rack designed to simultaneously flush 12 tubes was used. The rack consisted of a lower part to hold the tubes in place and an upper part that had a manifold with 12 outlets, each with a Pasteur pipet that bubbled N_2 through the media in the tube.

The contents of each tube were mixed using a vortex mixer (Sybron Maxi Mix model M-16715; Thermolyne, Dubuque, IA) for 30 s and then incubated at 55°C for 3 h. The tubes were centrifuged after incubation at 2150 rpm (975*g*) for 20 min, in order to separate the fungal cells from the liquid media. The supernatant was discarded and 2.5 mL of ethyl alcohol was added to the precipitated fungal cells. The tubes were vortexed and then centrifuged at 2150 rpm (975*g*) for 20 min. The supernatant (extracted formazan solution) was collected, and one more extraction of the precipitated fungal cells with 2.5 mL of ethyl alcohol was done. The supernatant was collected and the combined formazan solution was vortexed. Finally, the absorbance of the combined formazan solution was measured at 484 nm using a spectrophotometer (Spectronic 160; Fisher). The blank sample was used to zero the spectrophotometer. The formazan concentration (μmol/mL)

was determined from the standard curve. The total extracted formazan (μmol) was calculated as the formazan concentration (μmol/mL) multiplied by the amount of ethyl alcohol used for extraction (5 mL). Although 100% formazan recovery was not attained, the amount of formazan removed in the precipitated cells was insignificant and within the experimental error.

Experimental Design

Preliminary experiments conducted with *A. niger* vegetative cells according to the procedures described by Ghaly and Ben-Hassan *(28)* showed that little color was developed when samples were incubated for 2 h with TTC/glucose solution at a concentration of 5 g/L, a pH of 7.0, and a temperature of 24°C. Our work *(31)* showed that the optimum pH, temperature, and incubation time for measuring the dehydrogenase activity of *A. niger* using a TTC test were 9.0, 55°C, and 3 h, respectively. It was therefore necessary to investigate the effects of TTC concentration, O_2, and cell age on the development of TF at these optimum conditions. Two sets of experiments were conducted using *A. niger* vegetative cells previously grown in liquid media: The first was used to investigate the effect of TTC concentration and cell age on TF yield at the optimum pH, temperature, and incubation time; and the second was used to investigate the effect of O_2 on TF yield at the optimum TTC concentration, pH, temperature, and incubation time. All the laboratory ware used in these tests was sterilized at 121°C and 103.4 kPa for 30 min (Autoclave Model No. STM-E; Market Forge Sterilmatic). pH was measured using a Model 805MP pH meter (Fisher). Incubation took place in an Isotemp Oven (Model 655F; Fisher). The absorbance was measured at 484 nm using a Spectronic 601 spectrophotometer (Fisher).

Effect of TTC Concentration and Cell Age

The effect of TTC concentration (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, 25.0, 27.5, and 30.0 g/L) on color development was studied at the optimum test conditions (pH 9.0, 55°C, and 3 h of incubation) using *A. niger* vegetative cells from two cultures of different ages (4 and 12 d). For each sample tested, 0.1 g (wet basis) of *A. niger* vegetative cells was added to 4.7 mL of PDB and the TTC-test procedure was performed. Three replicates were carried out.

Effect of O_2

The TTC test was performed for four 4-d-old *A. niger* cell masses (0.025, 0.050, 0.075, and 0.1 g) under the optimum test conditions (TTC concentration of 20 g/L, pH 9.0, 55°C, 3 h of incubation) under both anaerobic (N_2 environment) and aerobic (O_2 environment) conditions. One set of sample tubes was flushed with nitrogen gas for 5 min before incubation, whereas aerobic conditions were maintained in the other set. Reduction of TTC under anaerobic conditions in the absence of living cells was not experienced in this work. Three replicates were carried out.

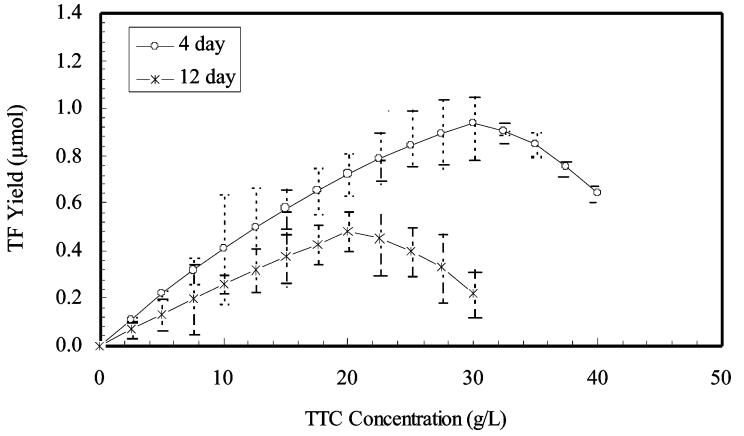


Fig. 2. Effects of TTC concentration and cell age on TF yield during measurement of dehydrogenase activity of *A. niger* under optimum conditions of pH, temperature, and incubation time.

Results and Discussion

Effects of TTC Concentration and Cell Age

Figure 2 shows the effect of TTC concentration on TF yield during measurement of dehydrogenase activity of *A. niger* vegetative cells of two different ages (4 and 12 d old) under the optimum conditions of pH, temperature, and incubation time. The values are the average of three replicates, and the coefficient of variation (CV) ranged from 1.99 to 25.17%. Our previous work *(31)* indicated that increasing the TTC concentration up to 15 g/L increased the TF yield exponentially. Since the rates of enzymatic reactions are affected by the substrate concentration *(34,37,38)* and the phenomenon of substrate inhibition has been well documented for biologic systems *(34,38–41)*, we decided to increase the TTC concentration beyond 15 g/L (up to 40 g/L) in order to study the toxicity effect of TTC on two cell groups of different ages. The results showed that increasing the TTC concentration initially increased the TF yield to a maximum value and then decreased it. The optimum TTC concentration (resulting in maximum TF yield) was 30 g/L (44.8 µmol) for young cells (4 d old) and 20 g/L (29.9 µmol) for old cells (12 d old). Further increases in TTC concentration above these optimum levels reduced the TF yield. The reduction in the TF yield caused by the higher concentration of TTC might be owing to the toxicity effect on the fungal cells.

The results showed that the cell viability, as measured by the amount of dehydrogenase enzymes present inside the fungal cells, was affected by cell age. The younger cells appeared to have more tolerance to toxicity. Shuler and Kargi *(34)* stated that the presence of toxic compounds in the medium results in the inactivation of cells or cell death and, consequently, the enzymatic activity is negatively affected. They also indicated that the

intracellular composition of cells varies depending on the type and age of cells and the composition of the medium. Tengerdy et al. *(33)* and Ohara and Saito *(35)* reported that the tetrazolium salts inhibit the growth of microorganisms to some extent. Griebe et al. *(30)* detected a significant reduction in formazan formation when the 5-cyano-2, 3-ditolyl tetrazolium chloride concentration was increased from 2 to 10 m*M* (0.62-3.12 g/L) and pointed out that this reduction indicates physiologic inhibition.

The relationship between the reaction rate and substrate concentration for enzymatic reactions is usually described by the following Michaelis-Menten kinetic equation *(37)*:

$$\upsilon = \frac{\upsilon_{\max C}}{K_m + C} \tag{4}$$

in which υ is the volumetric rate of reaction (μmol/[mL(h]), *C* is the concentration of the substrate (μmol/mL), υ_{max} is the maximum reaction rate at infinite substrate concentration and is proportional to the amount of active enzyme present (μmol/[mL(h]), and K_m is the Michaelis-Menten constant and is equal to the substrate concentration at half the maximum reaction rate (μmol/mL).

At low substrate concentrations, the reaction follows first-order kinetics in which the reaction rate increases with an increase in substrate concentration. At high substrate concentration, the reaction rate follows zero-order kinetics and approaches a constant maximum value independent of substrate concentration (Fig. 3). The values of and Km can be obtained graphically (Fig. 4) using a Langmuir plot by converting the Michaelis-Menten equation into the following linear form:

$$\frac{C}{\upsilon} = \frac{K_m}{\upsilon_{\max}} + \frac{C}{\upsilon_{\max}} \tag{5}$$

Doran *(37)* reported that the linearization using Langmuir plot minimizes distortions in experimental error. The maximum dehydrogenase activity (υ_{max}) and the Michaelis-Menten constant (K_m) were determined, and the volumetric rate of reaction for the young cells (υ_4) and the old cells (υ_{12}) can therefore be written as follows:

$$\upsilon_4 = \frac{0.95C}{88.55 + C} \tag{6}$$

$$\upsilon_{12} = \frac{1.12C}{177.41 + C} \tag{7}$$

Figure 5 shows that the previous equations do not account for the substrate inhibition. The substrate inhibition concentration (C_{in}) was 44.8 and 29.9 μmol for young (4 d) and 12-d-old cells, respectively. Shuler and Kargi *(34)* and Blanch and Clark *(38)* reported that some compounds (known as enzyme inhibitors) may bind to the enzymes, causing reduction in their activity. This nonproductive binding is known as substrate inhibition and has been described by several models, as shown in Table 1. The

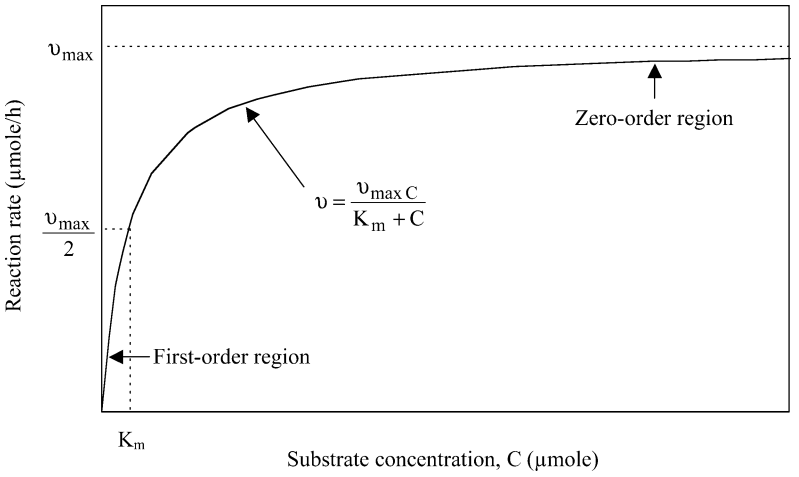


Fig. 3. Michaelis-Menten plot.

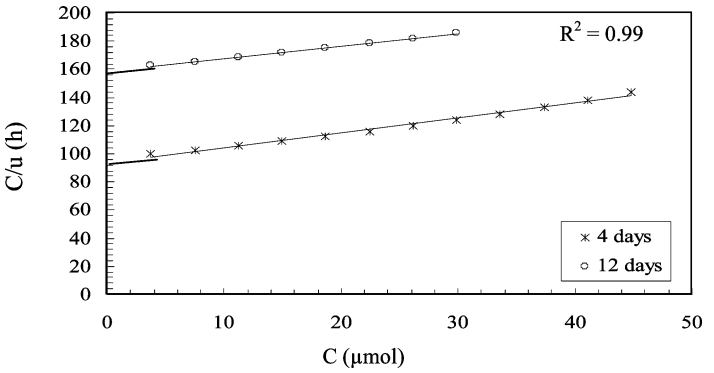


Fig. 4. Graphic determination of K_m and υ_{max} (Langmuir plot).

values of kinetic constants (υ_{max} and K_m) obtained from the Langmuir plot and inhibition concentration (K_i) obtained experimentally for the two cultures were used to test the validity of these models.

The model by Loung *(42)* deviated significantly from the measured data and was therefore excluded from the graph. None of the other models could describe the experimental data obtained from the current study, as shown in Fig. 6. Therefore, in order to better account for the substrate inhibition, the following new model was developed

$$\upsilon = \frac{\upsilon_{max} C}{K_m + C} \qquad (C \le C_{in})$$

$$= \frac{\upsilon_{max} C}{K_m + C} - K_{in}\left(C - C_{in}\right) \quad (C > C_{in}) \tag{13}$$

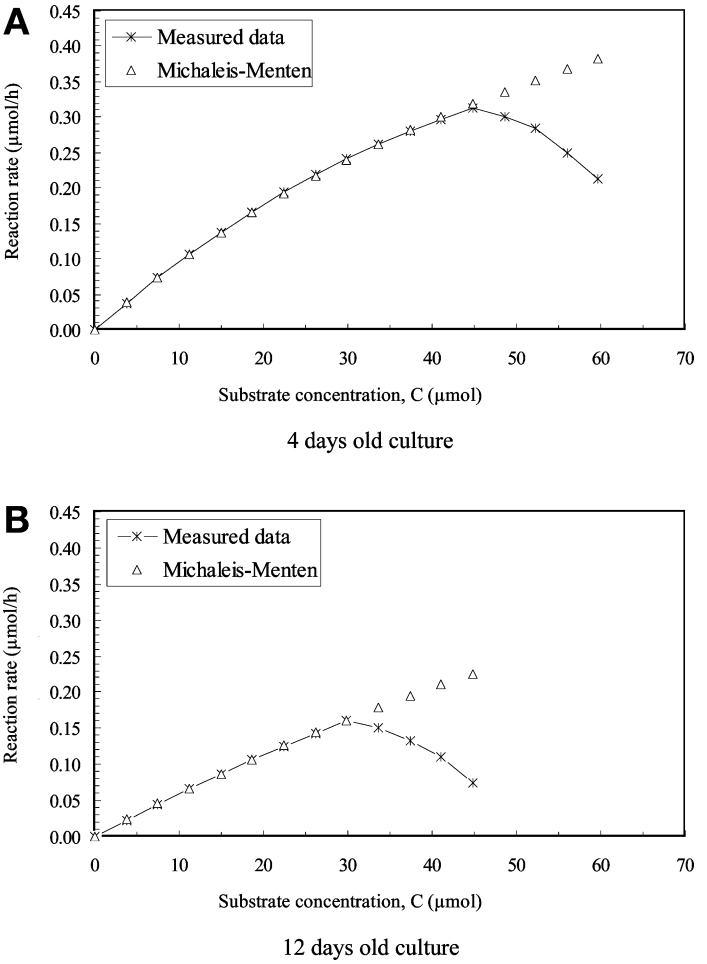


Fig. 5. Measured and predicted data using Michaelis-Menten model .

By plotting the values of ($\upsilon_{MM} - \upsilon_M$), which is calculated by subtracting the values of the measured reaction rate from the values of the calculated reaction rate obtained from the Michaelis-Menten kinetics equation, versus ($C - Cin$) values, a straight line with a slope of K_{in} and an intercept of zero was obtained (Fig. 7). The relationship (Fig. 8) between reaction rate and TTC concentration for the young (υ4) and old (υ12) cells can be described by the following equation:

$$\upsilon_4 = \frac{0.95C}{88.55 + C} \qquad (C \le C_{in})$$

$$= \frac{0.95C}{88.55 + C} - 0.0108\left(C - C_{in}\right) \qquad (C > C_{in}) \qquad (14)$$

$$(0\ \mu\text{mol} < C < 59.75\ \mu\text{mol})$$

Table 1
Substrate Inhibition Models

Reference	Model	
40	$$\upsilon = \frac{\upsilon_{\max} \cdot C\left(1 + \frac{C}{K_i}\right)}{K_m + C + \frac{C^2}{K_i}}$$	(8)
42	$$\upsilon = \frac{\upsilon_{\max} \cdot C}{K_m + C} - \left[1 - \left(\frac{C}{K_i}\right)^n\right]$$	(9)
34,38	$$\upsilon = \frac{\upsilon_{\max} C}{C \cdot \left(1 + \frac{C}{K_i}\right) + K_m}$$	(10)
43,44	$$\upsilon = \left(\frac{\upsilon_{\max} \cdot C}{K_m + C}\right)\left(\frac{K_i}{K_i + C}\right)$$	(11)
45	$$\upsilon = \frac{\upsilon_{\max} \cdot C}{K_m + C} \cdot e^{-(C/K_i)^n}$$	(12)

Effect of O_2

Figure 9 shows the TF yield during measurement of dehydrogenase activity of different masses of old *A. niger* vegetative cells under optimum test conditions (pH 9.0, 55ºC, 20 g/L TTC concentration, and 3-h incubation). The values are the average of three replicates and are presented on a dry weight basis. The CV ranged from 1.72 to 10.02%.

The relationship between the dehydrogenase activity of *A. niger* cells (as measured by the TF yield under optimum test conditions) and cell weight for both aerobic and anaerobic conditions was linear. Tengerdy et al. *(33)* reported a similar correlation between triphenyl formation yield of *Escherichia coli* and S*taphylococcus aureus* and their viable plate counts. Ghaly and Ben-Hassan *(28)* obtained a linear relationship between TF yield (μmol) and concentration (mg/L) of active cells of *Kluyveromyces fragilis* and *Candida pseudotropicalis*.

The results of the present study showed that the TF yield for young and old cells under anaerobic conditions (4.01 μmol of TF/g of cells and 1.2 mg of TF/g of cells) was higher than that under aerobic conditions (3.29 μmol of TF/g of cells and 0.96 mg of TF/g of cells). Tetrazolium salts act as electron acceptors from the oxidized substrate or coenzyme. In the absence of tetrazolium salts, these electrons would normally pass via a series of carriers to a physiologic acceptor, which in most cases is oxygen.

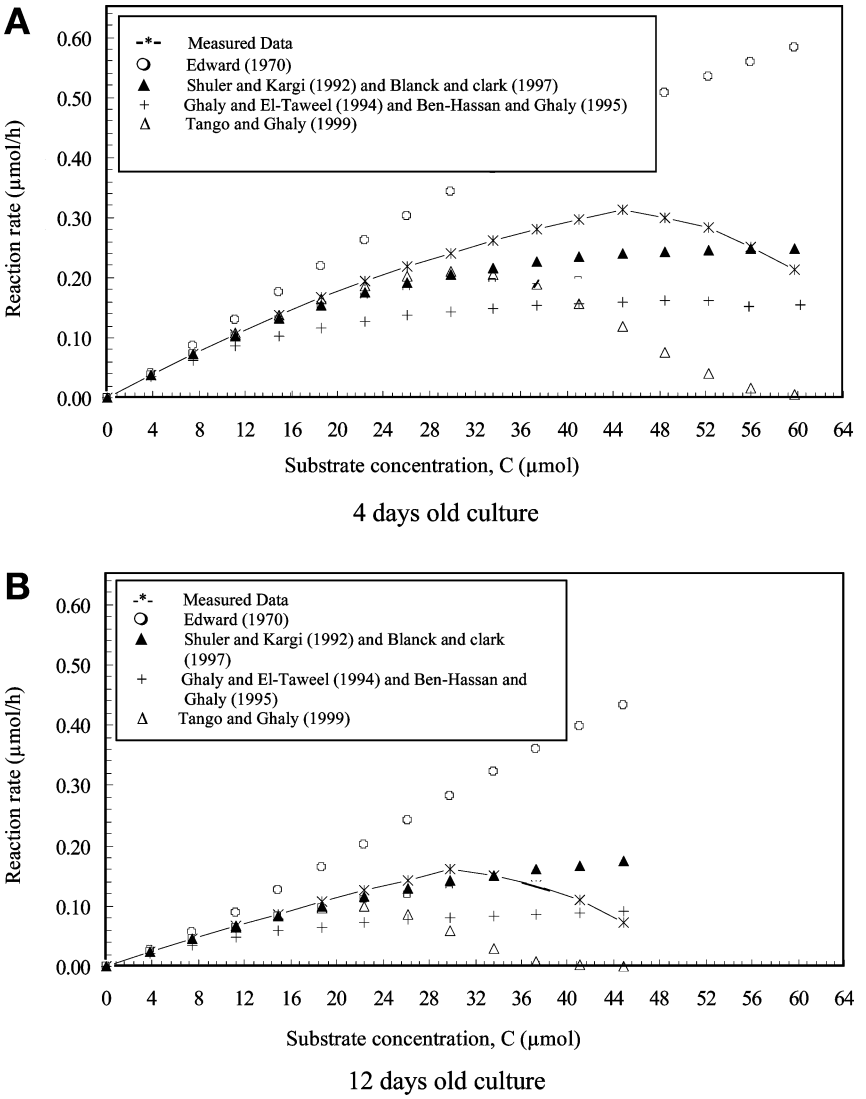


Fig. 6. Measured and predicted data using various models.

Ross *(46)* and Trevors *(47)* reported that a TTC test gives significantly higher formazan formation in systems incubated anaerobically than in systems incubated without complete anaerobiosis. Altman *(48)* proposed that one electron reduction of TTC results in TTC free radical, which under anaerobic conditions is reduced further by the addition of another electron to TF. Under aerobic conditions, the TTC radical reduces molecular oxygen to the superoxide radical $O^{\cdot}_2$ and itself becomes oxidized back to TTC. In the present study, about 18% of the TF produced under aerobic conditions was converted back into TTC.

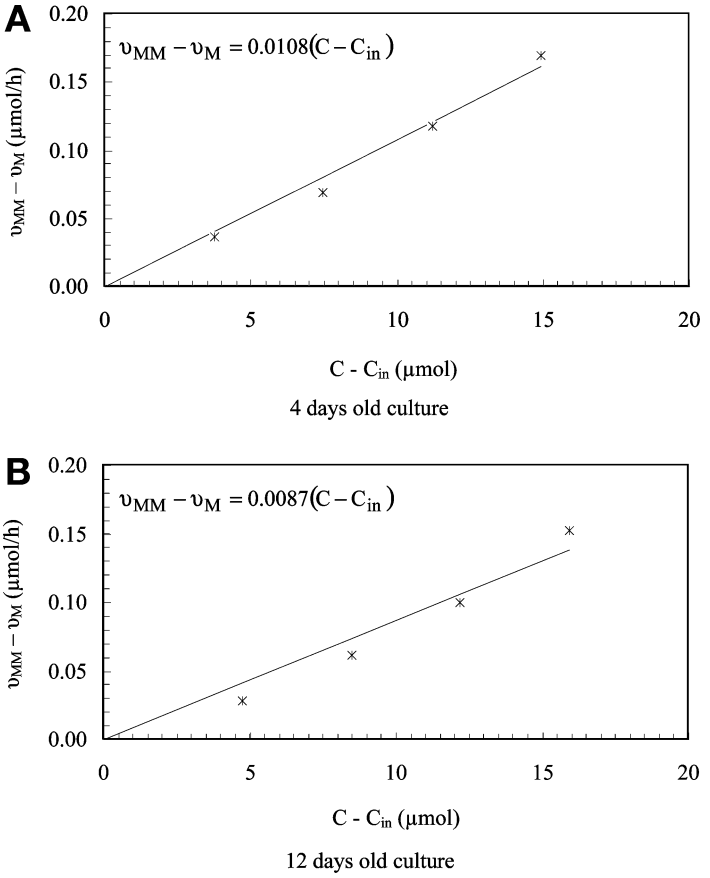


Fig. 7. Graphic determination of inhibition constant (K_{in}): **(A)** 4-d-old culture; **(B)** 12-d-old culture.

Conclusion

TTC test can effectively be used for quantifying the growth of the fungal species *A. niger*. A linear relationship between the dehydrogenase activity of *A. niger* and cell mass was observed. Increasing TTC concentration initially increased the TF yield and then caused inhibition. Cell tolerance to high TTC concentration was dependent on cell age. The optimum TTC concentration (which resulted in maximum TF yield) was 30 g/L for young cells (4 d old) and 20 g/L for old cells (12 d old). Conducting the TTC test under anaerobic conditions increased the TF yield. About 18% of the TF produced under aerobic conditions was converted back into TTC. The optimum TTC test conditions for dehydrogenase activity measurements of A. niger were a TTC concentration of 20 g/L, a pH of 9.0, a temperature of 55°C, an incubation time of 3 h, and an anaerobic environment. A kinetic

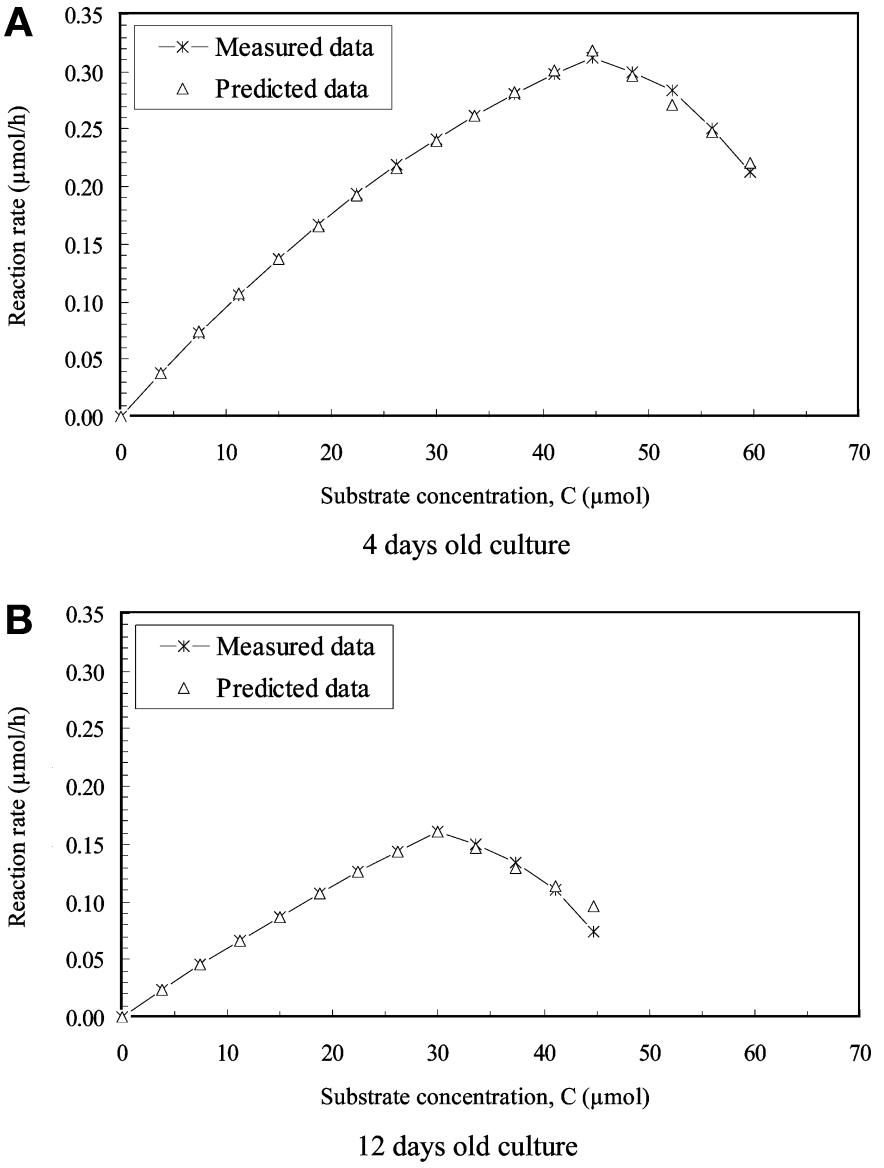


Fig. 8. Measured and predicted substrate concentrations: **(A)** 4-d-old culture; **(B)** 12-d-old culture

model that describes the relationship between reaction rate (µmol of TF formed/h) and TTC concentration while accounting for substrate inhibition was developed and the model constants were calculated. *A. niger* is used in various biotechnology applications for the production of industrially important products. Using the TTC test for quantifying growth would facilitate on-line monitoring and control of the microorganisms in bioreactors.

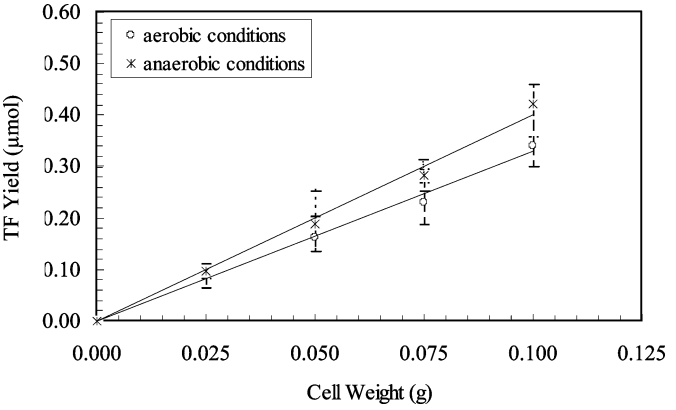


Fig. 9. Correlation between dehydrogenase activity (as measured by total formazan yield under optimum conditions of TTC concentration, pH, temperature, and incubation time) and mass of *A. niger* vegetative cells.

Acknowledgment

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