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Effect of assay conditions on the measurement of dehydrogenase activity of *Streptomyces venezuelae* using triphenyl tetrazolium chloride

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ABSTRACT

Jadomycin is an antibiotic that has shown activities against bacteria, yeasts and fungi as well as cytotoxic properties to cancer cells. Because of the wide range of its inhibitory actions, jadomycin shows promise as a novel antibiotic and cancer treatment drug. *Streptomyces venezuelae* are aerobic bacteria that produce jadomycin and the size of bacterial population can significantly affect the yield of jadomycin. Therefore, the bacterial population must be accurately measured in order to standardize the reproducibility of jadomycin production process. In this study, a dehydrogenase activity measurement test, using triphenyl tetrazolium chloride (TTC), was used to measure the dehydrogenase activity of *Streptomyces venezuelae* during growth in maltose-yeast extract-malt extract (MYM) broth. The aims were to evaluate the effectiveness of the test for measuring microbial growth and to study the effects of the test conditions (incubation time, incubation temperature and medium pH) on triphenyl formazan (TF) yield. The results showed that the TF yield was highly correlated to the optical density. The highest TF yield was observed at a pH of 6 at all incubation times and temperature. Lower TF yields were obtained at higher temperature (40˚C and 50˚C) compared to those obtained at lower temperatures (22˚C and 30˚C). The difference between the yields obtained at 22˚C and 30˚C were not significant. The differences between incubation time were also not significant. The recommended test conditions are an incubation time of 1 hour at a temperature of 30˚C and a pH of 6 followed by three extractions using methanol.

***Keywords*:** Dehydrogenase Activity; Growth; Triphenyl Tetrazolium Chloride (TTC); Triphenyl Formazan; Jadomycin; *Streptomyces venezuelae*; Temperature; pH; Incubation Time

1. INTRODUCTION

Jadomycins are type II polyketide synthesis-derived secondary metabolites produced by the actinomycete *Streptomyces venezuelae* [1]. Jadomycins have demonstrated antibacterial, antitumor, antifungal and enzyme inhibitory functions as well as cytotoxic properties to the cancer cells [2]. They are considered to be promising novel antibiotics and cancer treatment drugs [3]. The production of jadomycin takes place in a nutrient-deprived (exhaustion of carbon, nitrogen or phosphate from the culture medium) amino acid rich environment assisted by environmental shock using ethanol or heat [4]. The formation of the cyclized product of jadomycin is due to the presence of amino acids in the culture medium which has a biosynthetic aldehyde precursor that generates a reactive aldimine to form jadomycin [5]. The production yield of jadomycin has been extensively linked to the mass of vegetative cells of *Streptomyces venezuelae* that is transferred from the growth medium to production medium [6]. Therefore, it becomes essential to determine the viable cell mass of *Streptomyces venezuelae* in growth media for the improvement and regularization of the reproducibility of the jadomycin production process.

Dehydrogenase activity measurement can be used for the determination of bacterial growth and metabolism [7]. The test is based on the fact that the dehydrogenase enzymes are produced by all living cells and the extend to which the enzymes oxidize organic matter can be related to the number of living cells during the growth phase and their activities during the production phase [8]. The dehydrogenase enzymes transport electrons and a hydrogen atom from an oxidized matrix to an electron acceptor [9,10].

$$\underset{\text{substrate}}{RH_2} \xrightarrow{\text{Dehydrogenase}} \underset{\text{Oxidized substate}}{R} + 2H^+ + 2e^- \quad (1)$$

The dehydrogenase activity is largely measured using the triphenyl tetrazolium chloride (TTC) salt as a hydrogen acceptor [11,12]. TTC is a colourless salt which turns to a red triphenyl formazan (TF) dye as soon as it accepts hydrogen atoms. The TTC activity is determined colorimetrically by measuring the irreversible colour change (red color) during the reaction.

Different solvents are used for the extraction of TF from cells and the TF concentration is determined by measuring the optical density. The TF produced from the test is used as a measure of the amount of the living cells [8,13-15]. However, TF yield is depends upon several factors: type of solvent, number of extractions, incubation time, incubation temperature, and medium pH [16]. The purpose of this study was to evaluate the effects of assay conditions (medium pH, incubation time and incubation temperature) on dehydrogenase activity and the TF yield from *S. venezuelae*.

2. MATERIALS AND METHODS

2.1. Reagents

Tris (hydroxymethyl)-aminomethane and triphenyl tetrazolium chloride (TTC) were used to measure the dehydorogenase activity. A TTC-glucose reagent (1 g glucose and 2 g TTC dissolved in 100 mL distilled water) was prepared and stored in the dark at 4˚C until used. Triphenyl formazan (TF) dye was used to establish a standard curve for absorbance (OD_{484}) vs. TF concentration. NaOH and HCl were used to adjust the pH of the sample. Ethanol and methanol were used to extract the TF from bacterial cells. The Tris, TTC, TF, ethanol, methanol, NaOH and HCl were obtained from Sigma-Aldrich (Oakville, Ontario, Canada) and glucose was obtained from BioShop (Burlington, Ontario, Canada). Ethanol and methanol were obtained from Fisher Scientific (Montreal, Quebec, Canada).

2.2. Media Preparation

Maltose-yeast extract-malt extract (MYM) agar and broth were used to cultivate Streptomyces venezuelae. The composition of MYM agar and broth are shown in

Table 1. MYM media components.

Component	Quantity(g/L distilled water)	
	Agar	**Broth**
Maltose	4.0	4.0
Yeast Extract	4.0	4.0
Malt Extract	10.0	10.0
MOPS	1.9	1.9
Agar	15.0	-

Table 1. All media components were obtained from BioShop (Burlington, Ontario, Canada). The media components were dissolved in the distilled water then autoclaved (SterileMax, Harvey Thermo Fisher Scientific, Ottawa, Ontario, Canada) on the liquid setting (121˚C, 20 Pa) for 15 minutes. The autoclaved agar was stored at 65˚C to prevent solidification

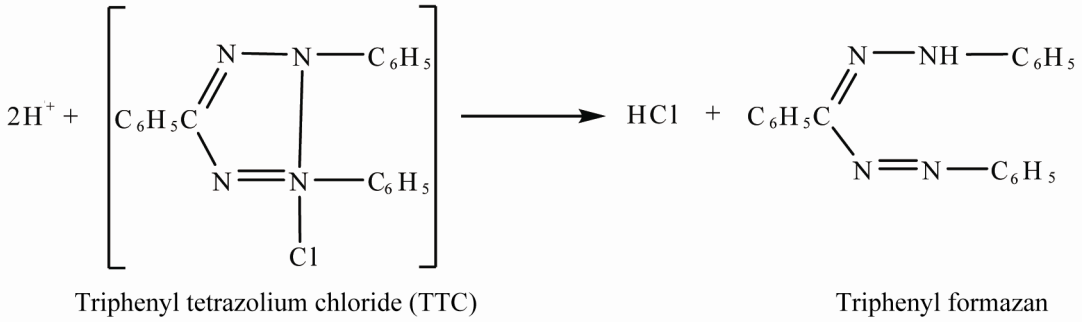
2.3. Bacteria

An initial starter plate of *Streptomyces venezuelae* ISP5230 was obtained from the Brooks Biotechnology Laboratory, Faculty of Engineering, Dalhousie University (Halifax, Nova Scotia, Canada) and stored at 4˚C. The surface growth was used to inoculate maltose-yeast extract-malt extract (MYM) agar plates or flasks with MYM broth.

2.4. Triphenyl Formazan (TF) Standard Curve

A standard curve was developed to determine the concentration of TF (µmol/mL) corresponding to an absorbance measurement at 484 nm (OD484). A stock solution of 0.2 µmol/mL was prepared by dissolving 0.03 g TF in 500 mL methanol. The stock solution was diluted with methanol to produce 11 solutions with TF concentrations ranging from 0.004 to 0.10 µmol/mL. The absorbance of each solution was measured with a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada) at a wavelength of 484 nm. The absorbance readings (OD_{484}) were plotted against the TF concentration (µmol/mL) as shown in **Figure 1**. The following linear best-fit Equation (R^2 = 0.98) was obtained:

$$OD_{484} = 10.574\ \mathrm{TF} \quad (3)$$



(2)

Triphenyl tetrazolium chloride (TTC) Triphenyl formazan

where:

OD_{484} is the absorbance reading at 484 nm (AU)

TF is the concentration of triphenyl formazan (µmol/mL of extraction solvent)

2.5. Bacterial Growth

Three 250 mL shake flasks were filled with 175 mL of MYM broth, plugged with foam caps, covered with aluminum foil and autoclaved (SterileMax, Harvey Thermo Fisher Scientific, Ottawa Ontario, Canada) for 15 minutes at 121˚C and 20 Pa. The flasks were then inoculated with *S. venezuelae* and incubated in a controlled environment shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 30˚C and 250 rpm. Each flask was sampled at 0, 2, 12, 14, 17, 20, 22, 37, 40, 42, 62 and 64 hours after inoculation and the extent of cell growth was monitored over a period of 64 hours by measuring the optical density at 600 nm (OD_{600}) and the dehydrogenase activity (TF yield).

To measure the dehydrogenase activity at each sampling time, an aliquot of 1 mL was transferred from each flask into each of the four test tubes (three replicates and control). The pH was adjusted to 7.5 using 1 N NaOH or HCl. Tris buffer (2.5 mL) was added to the all test tubes. This was followed by 1 mL of TTC/glucose solution added to the three sample test tubes whereas 1 ml distilled water added to control tube. The tubes were gently swirled to mix the contents, incubated at 50˚C for 1 hour in a temperature controlled oven (Isotemp Oven, Model 630F, Fisher Scientific, Ottawa, Ontario, Canada) and then centrifuged (IEC Centra CL2, Thermo Electron Corporation, Mississauga, Ontario) for 10 minutes to separate the cells from the liquid media. The supernatant was discarded and 2.5 mL of methanol was added to the cells in the tubes. All tubes were vortexed (Thermolyne Maxi Mix, Thermolyne Corporation, Hampton, New Hampshire, USA) to aid in the extraction of TF from the cells. Samples were centrifuged again and the supernatant decanted. Two more extractions with methanol were carried out as recommended by Burdock [17] and the supernatants were combined with that from the first extraction. The absorbance of three combined supernatant extractions was measured at 484 nm in a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada). The control test tube was used to adjust the spectrophotometer to zero. The TF yield was then determined using **Eq.3**.

2.6. Evaluating the Test Parameters

Experiments were conducted to determine the effect of test conditions on the reduction of TTC to TF and the optimum combination that allow the extraction of the highest amount of TF from *S. venezuelae* during growth in MYM media. The dehydrogenase activity assay pa-

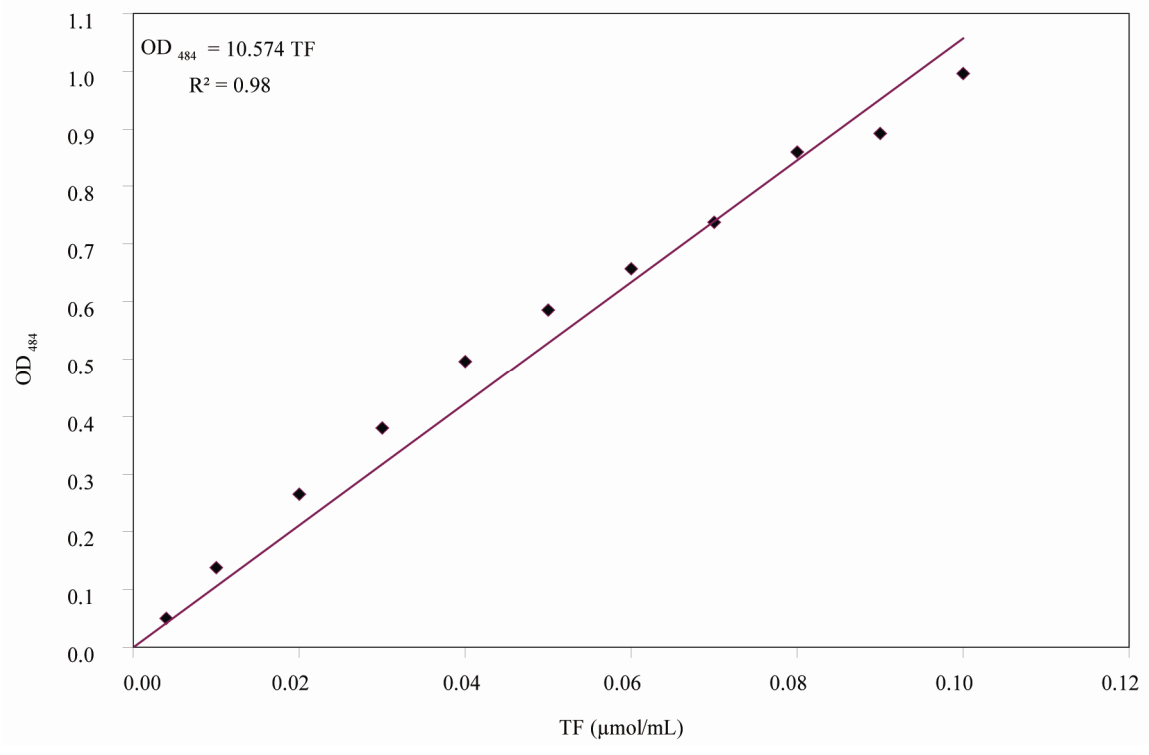


Figure 1. Standard curve relating TF concentration to absorbance at 484 nm (n = 3).

rameters investigated were: incubation time (1, 2, 3 and 4 h), incubation temperature (22˚C, 30˚C, 40˚C and 50˚C and medium pH (6.0, 7.5 and 9.0). Three extractions with methanol were carried out as recommended by Burdock [17]. The experiments were carried out in completely randomized factorial design (3 × 4 × 4) with three replicates, resulting in 144 experimental runs. The contents of all flasks were collected after 64 hours of growth, combined in a 1 L flask and refrigerated at 4˚C until required. For a given incubation temperature-medium pH—incubation—time combination, a 1 mL aliquot of the MYM broth containing *S. venezuelae* growth was added to each of four test tubes (three replicates and a control). The pH was adjusted to the desired value (6, 7 or 9) using 1 N HCl or NaOH. Tris buffer (2.5 mL) and TTC/glucose solution (1 mL) were added to each of the three sample test tubes whereas 1 ml distilled water was added to control tube. The tubes were manually swirled to mix contents and incubated for the assigned time (1, 2, 3 or 4 hours) at the desired temperature (22˚C, 30˚C, 40˚C or 50˚C). Tubes were incubated at 22˚C or 30˚C in an environment controlled incubator (Model 2020, VWR International, Cornelius, Oregon, USA) and at 40˚C or 50˚C in a temperature controlled oven (Isotemp Oven, Model 630F, Fisher Scientific, Ottawa, Ontario, Canada). Samples were then centrifuged for 10 minutes to separate the cells from the liquid media. The supernatant was discarded and extraction of TF from the cells was carried out using 2.5 mL methanol. Three extractions were carried out and the supernatants from the three extractions were combined. The absorbance was then measured at 484 nm in a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada). The control test tube was used to adjust the spectrophotometer to zero. The TF yield was then determined using **Eq.3**.

3. RESULTS

3.1. Bacterial Growth

The growth of *S. venezuelae* as measured by the optical density at 600 nm (OD_{600}) and the triphenyl formazan yield (TF) is presented in **Figure 2**. The results showed an initial lag period followed by exponential growth. The lag period was required for the bacteria to adjust to the new environmental condition and to produce the enzyme required for the utilization of growth substrate in the medium. The lag period and specific growth rate were determined graphically according to the procedure described by Ghaly *et al*. [18] as shown in **Figure 3**. The lag period and specific growth rate were 10.3 hours and 0.3 h^{-1} respectively.

3.2. Assay Parameters

Figure 4 shows the effect of medium pH on the TF yield at varying incubation times and incubation temperatures. All plots displayed similar shapes with the pH value of 7.5 resulting in the lowest TF yield and the pH value of 6 resulting in the greatest TF yield. **Figure 5** shows the effect of incubation time on the TF yield at varying incubation temperatures and medium pH values. The results showed slight increases in TF yield at longer incubation time. **Figure 6** shows the effect of incubation temperature on the TF yield at different incubation times and medium pH values. The results showed that higher incubation temperatures (40˚C and 50˚C) resulted in lower TF yields than these obtained at lower incubation temperatures (22˚C and 30˚C).

Table 2 shows the analysis of variance performed out on the TF yield data. The medium pH, incubation time and incubation temperature had significant effects on TF yield ($P < 0.001$) with pH 6 having the highest TF yield. The two-way interactions (pH-Time, pH-Temperature and Time-Temperature) were also significant ($P < 0.008$) but the three-way interaction was not significant. **Table 3** shows the results of Duncan multiple range test performed on the TF data. All three levels of the medium pH were significantly different from one another ($P < 0.05$) with pH 6 having the highest TF yield. The incubation temperatures of 22˚C and 30˚C were not significantly different from one another. Also, the incubation temperatures of 40˚C and 50˚C were not significantly different from one another. However, the lower temperatures (22˚C and 30˚C) had significantly higher TF yield than the higher temperatures (40˚C and 50˚C). All the four incubation times were not significantly different from one another.

4. DISCUSSION

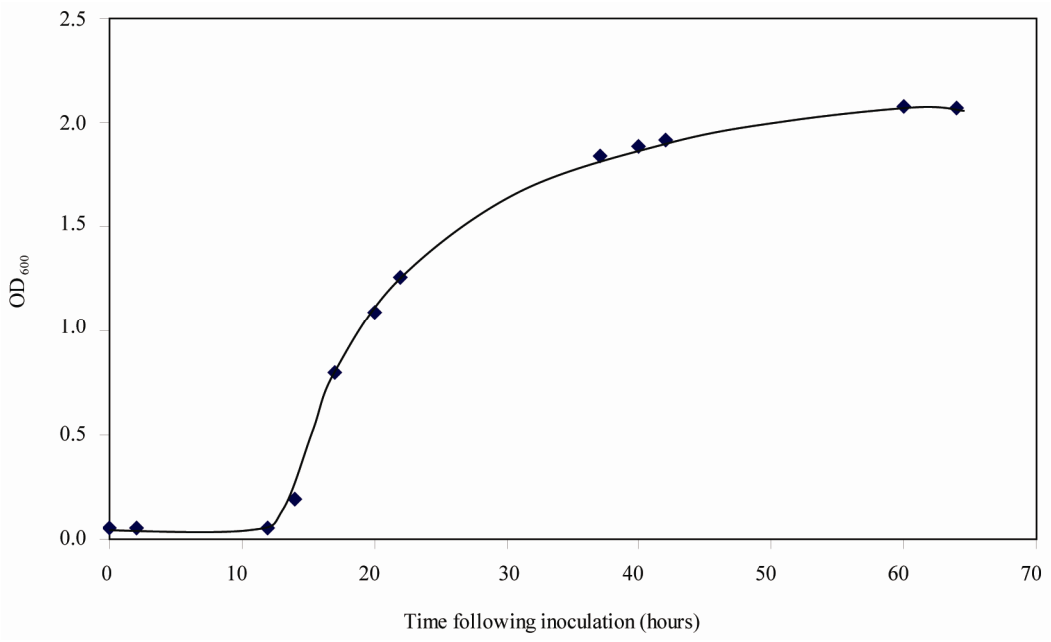
4.1. Bacterial Growth and Activity

Jakeman *et al.* [6] monitored *S. venezuelae* population during the growth period by measuring the optical density at 600 nm (OD_{600}). In this study, the change of *S. venezuelae* population during the growth period was

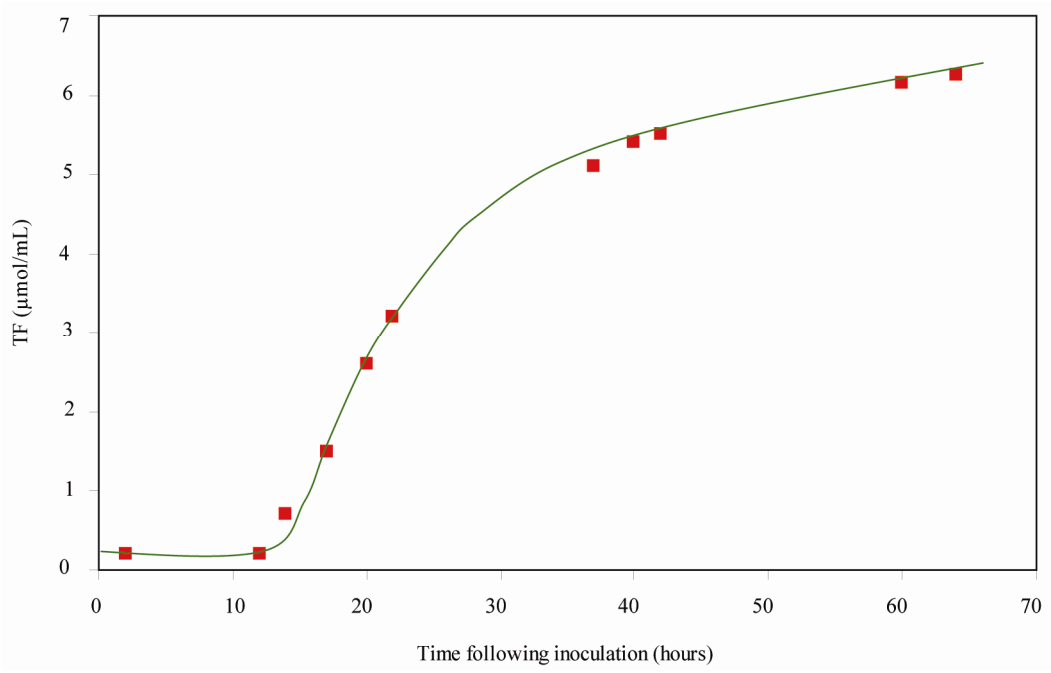
Table 2. Analysis of variance for dehydrogenase activity test parameters.

Source	DF	SS	MS	*F*-Value	*P*
Total	143	49.5667			
Model	47	40.0942			
pH	2	10.9850	5.493	61.134	0.0001
t	3	1.6077	0.536	5.961	0.0010
T	3	18.0770	6.026	67.066	0.0001
pH × t	6	1.9600	0.327	3.636	0.0030
pH × T	6	3.7340	0.622	6.927	0.0001
t × T	9	2.3770	0.264	2.939	0.0040
pH × t × T	18	2.2010	0.122	1.361	0.1700
Error	96	8.6250	0.090		

pH = Medium pH; t = Incubation Time (hours); T = Incubation Temperature (˚C); $R^2 = 0.83$.



(a)



(b)

Figure 2. *S. venezuelae* growth in nutrient rich MYM broth as measured by optical density and TF yield.

monitored by measuring the optical density at 600 nm (OD_{600}) and the triphenyl formazan yield (TF) (**Table 4**). The relationship between OD_{600} and TF is presented in **Figure 7**. The amount of TF extracted was correlated (R^2 = 0.9833) with OD_{600}.

The specific growth measured in this study was 0.3 h^{-1}. Abdel-Fattah [19] reported maximum specific growth rates of 0.57 and 0.23 h^{-1} for *S. venezuelae* grown in media containing glucose and soluble starch at 30˚C, respectively. Glazebrook *et al.* [20] reported a maximum specific growth rate of 0.14 h^{-1} for S. venezuelae grown in MYM medium at 27˚C.

4.2. Assay Parameters

A significantly acidic pH (1.5 - 3) was found to result

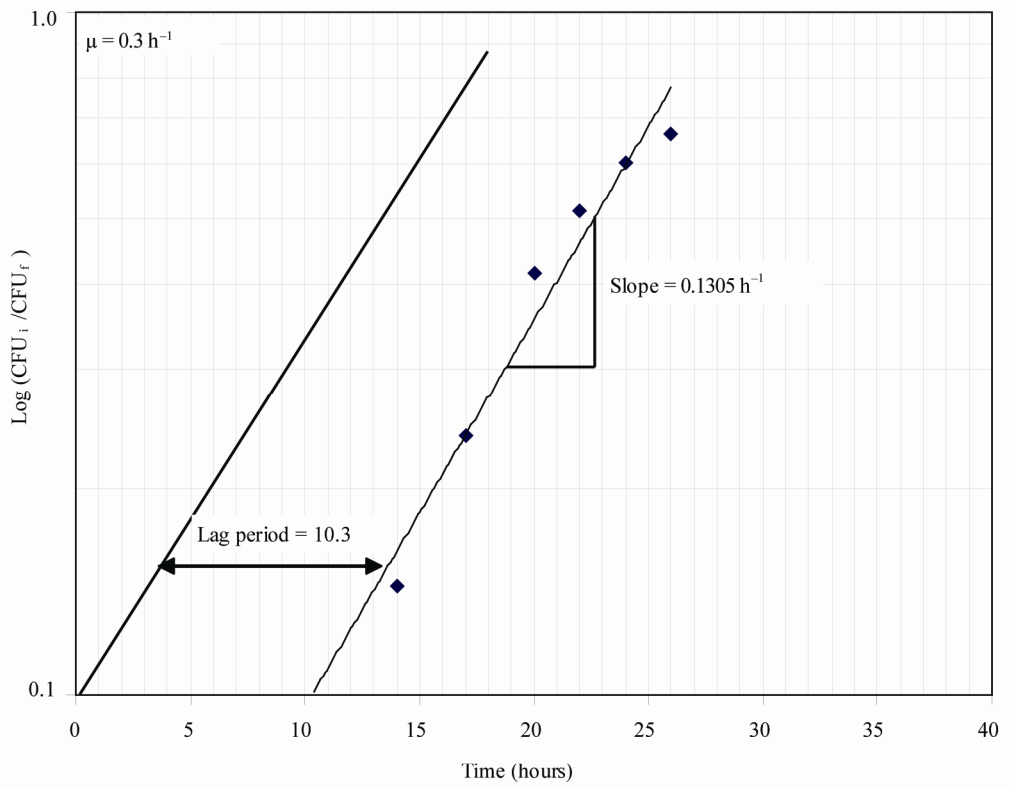


Figure 3. Graphical determination of the lag period and specific growth rate.

Table 4. Means of TF yield for pH, incubation temperature and incubation time.

Parameter	Number of observations	Mean of TF yield	Duncan Grouping*
pH			
6.0	48	1.195	A
7.5	48	0.525	B
9.0	48	0.780	C
Incubation Temperature (˚C)			
22	36	1.240	A
30	36	1.101	A
40	36	0.632	B
50	36	0.359	B
Incubation Time (hours)			
1	36	0.693	A
2	36	0.804	A
3	36	0.843	A
4	36	0.988	A

*Means with different letters are significantly different from one another at 95% confidence level.

in higher TTC reduction in lichens [21]. No other reports for higher TF yields at acidic pH were found in the literature. However, higher TF values are always associated with natural or slightly alkaline conditions. Mahmoud and Ghaly [13] found that at a pH of less than 7, no reduction of TTC occurred for *Kluyveromyces fragilis* growing in cheese whey and mixed culture growing in compost materials. Jones and Prasad [22] measured sludge activity and noticed a considerable amount of TTC reduction at pH 7.6 compared to pHs 3.2 and 5.3. Mersi and Sehinne [23] reported optimal iodonitrotetrazolium chloride (INT) reduction in soils at pH 7.0. Brzezińska *et al*. [24] observed maximum dehydrogenase activity in soils at pH of 6.6 - 7.2. Ghaly and Ben-Hassan [25] found maximum dehydrogenase activities for both *Kluyveromyces fragilis* and *Candida pseudotropicalis* yeast at a pH of 7 and lower activities at the more acidic and basic levels of pH. Higher TF yield was observed at a pH of 9 for *A. niger* by Ghaly and Mahmoud [26]. However, at high pH values non-enzymatic reduction of TTC to TF may also occur [13]. It is not clear if non-enzymatic reduction of TTC to TF occurred at a pH of 9 in this study. The results indicated that a pH of 6 is the most appropriate value for measuring dehydrogenase activity in *S. venezuelae* during growth in MYM broth.

Several investigators reported that incubating samples for longer times increased the extent of TTC reduction to TF. Mahmoud and Ghaly [27] reported that TF yield increased exponentially with incubation time for *A. niger* in the range of incubation periods studied (1.5 - 4.5 hours). Ghaly and Ben-Hassan [25] found that the TF yield increased with increased incubation time for both *Kluyveromyces fragilis* and *Candida pseudotropicalis* yeast cells, but appeared to plateau after about 80 hours in

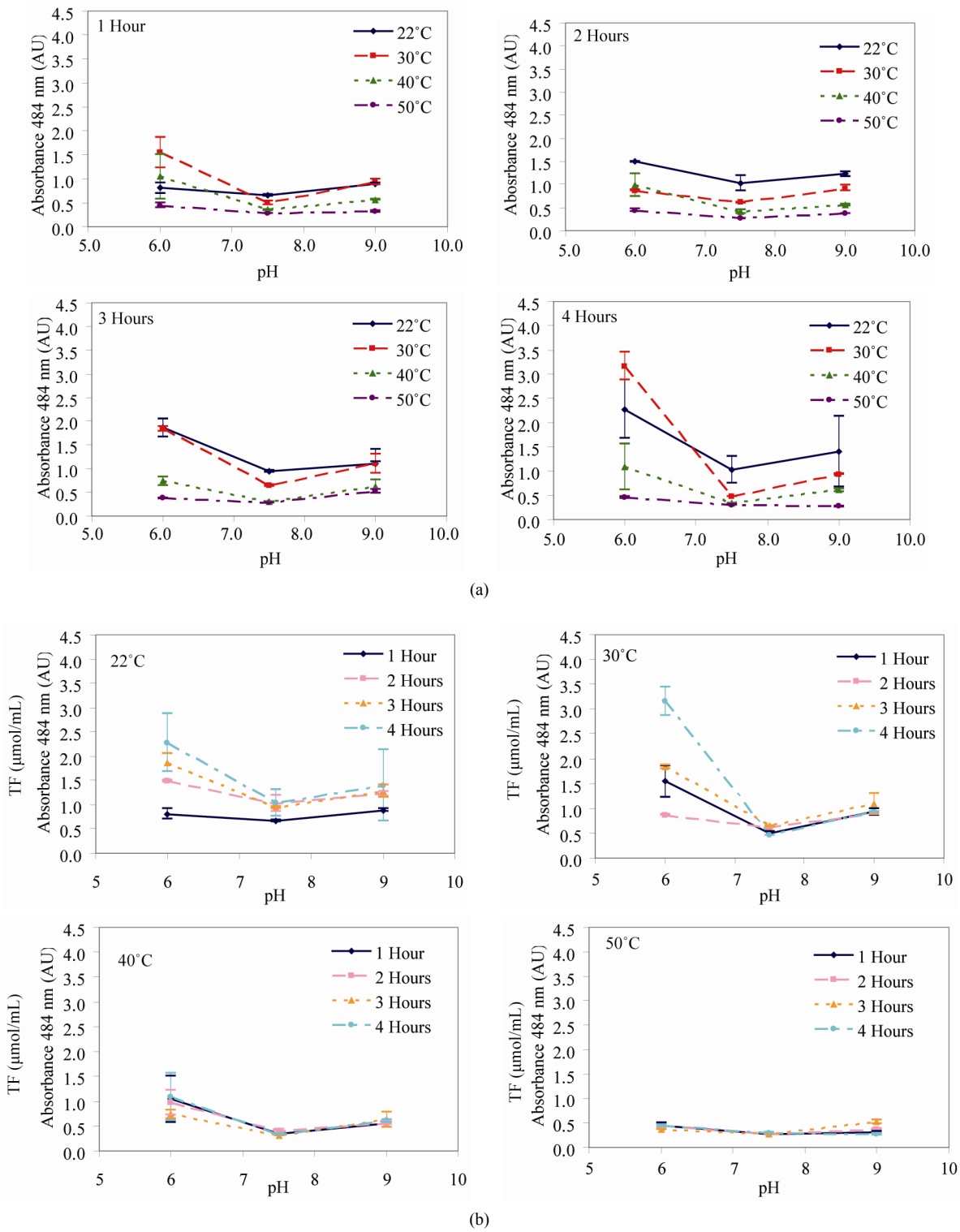


Figure 4. Effect of pH on triphenyl formazan formation (TF) at various incubation temperatures and times. (a) Incubation Time; (b) Incubation Temperature.

both cases. Mathew and Obbard [28] reported increasing INT-formazan yield with increased incubation time for petroleum-contaminated beach sediments and observed leveling off of TF yield after 22 hours of incubation. Tengerdy *et al.* [29] reported increasing TF yield of *E. coli* and *S. aureus* cultures during a 30 hours incubation

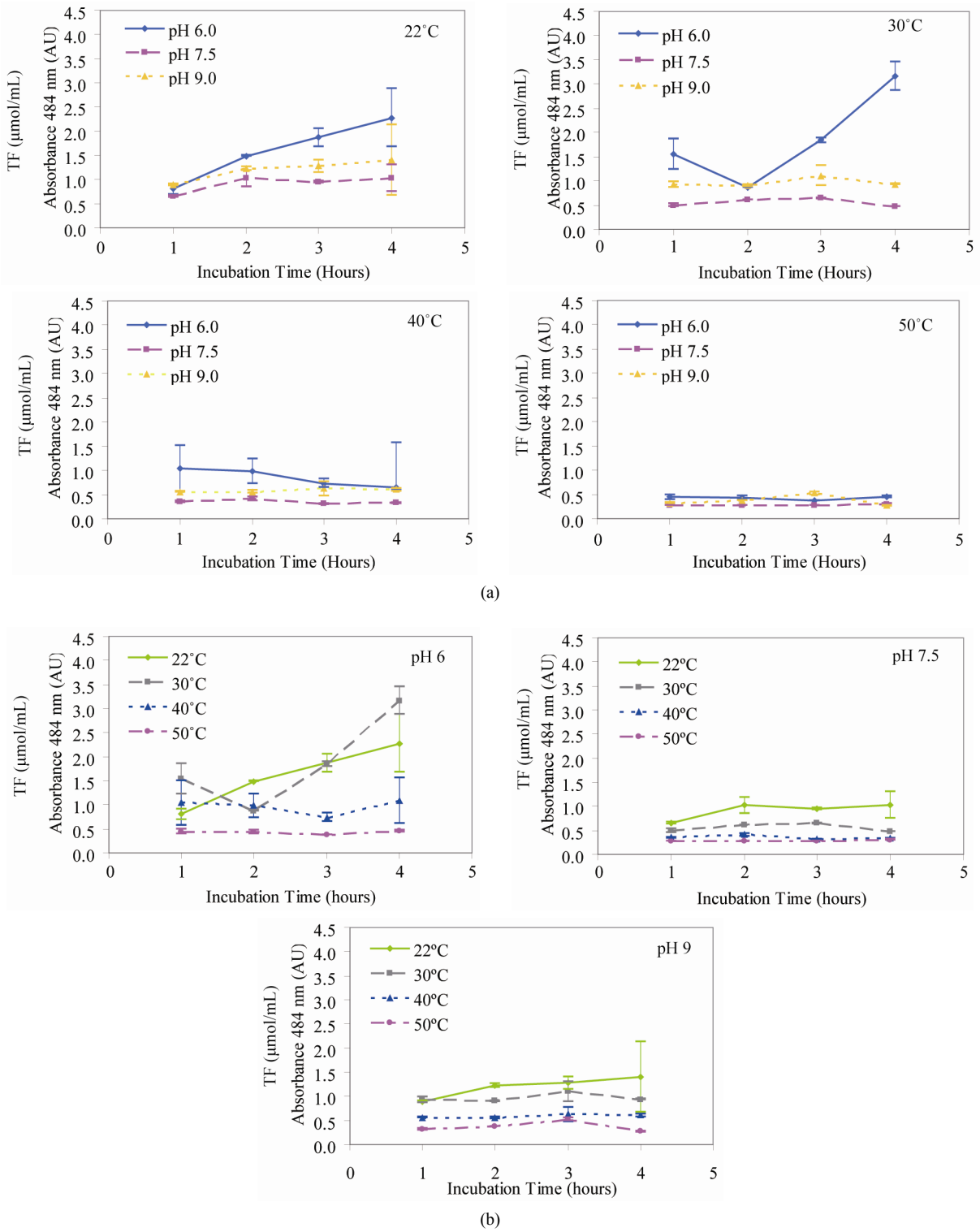


Figure 5. Effect of incubation time on triphenyl formazan (TF) formation at various incubation temperatures and medium pH values. (a) Incubation Temperature; (b) Medium pH Value.

period and observed stationary yield of TF after 30 hours. Griebe *et al.* [30] incubated activated sludge for a period of 24 hours with redox-sensitive dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and found no further increase in fromazan yield after 2 hours of incubation. In this study, a slight increase in TF yield from *S. venezue-*

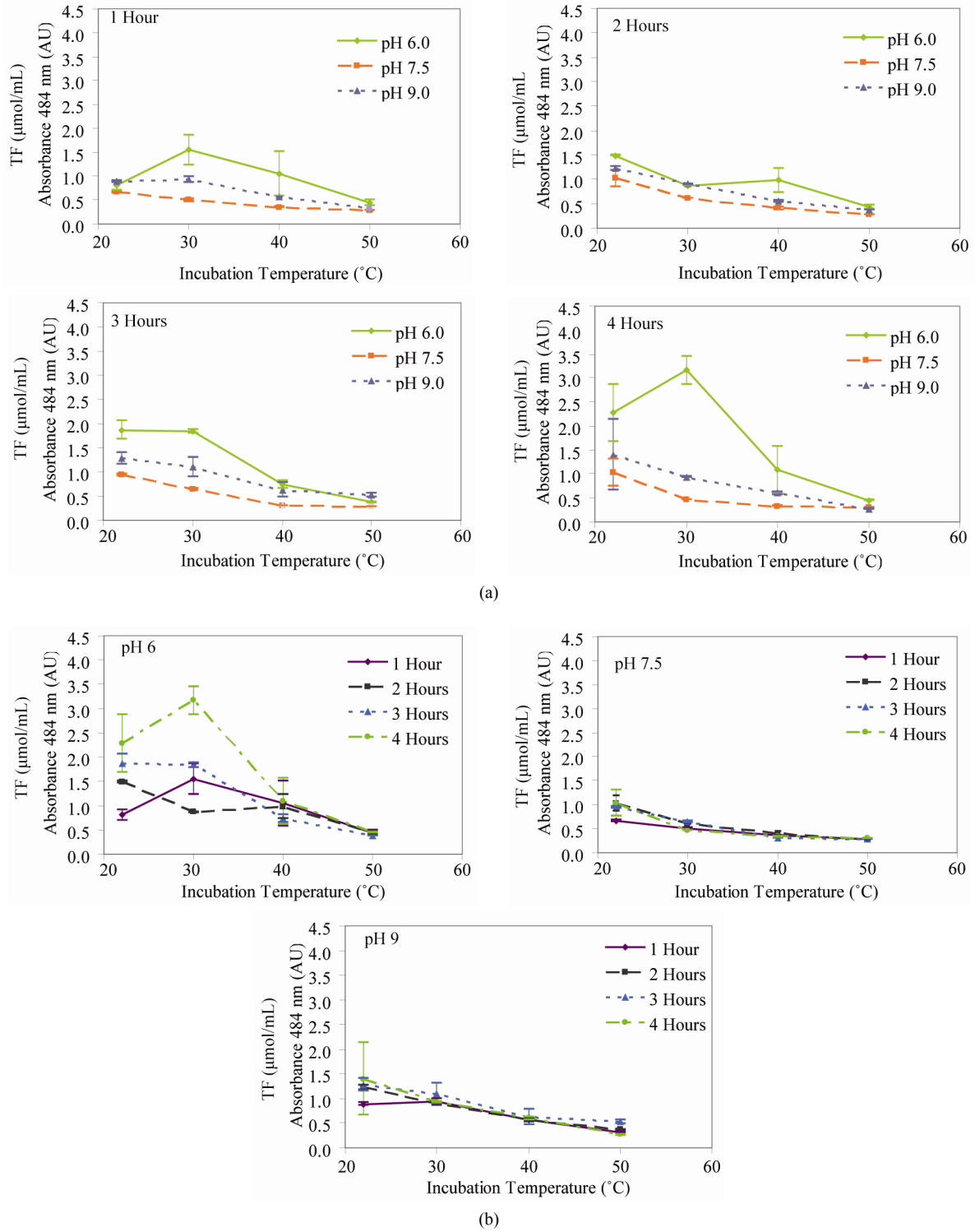


Figure 6. Effect of incubation temperature on triphenyl formazan (TF) formation at different incubation times and medium pH values. (a) Incubation Time; (b) Medium pH Value.

lae was observed when the incubation time was increased from 1 to 4 h. It also, appears that longer incubation times are required for lower temperatures (22˚C and 30˚C) compared to those required for higher temperatures (40˚C and 50˚C). However, the increases in TF yield observed in this study due to increase in incubation

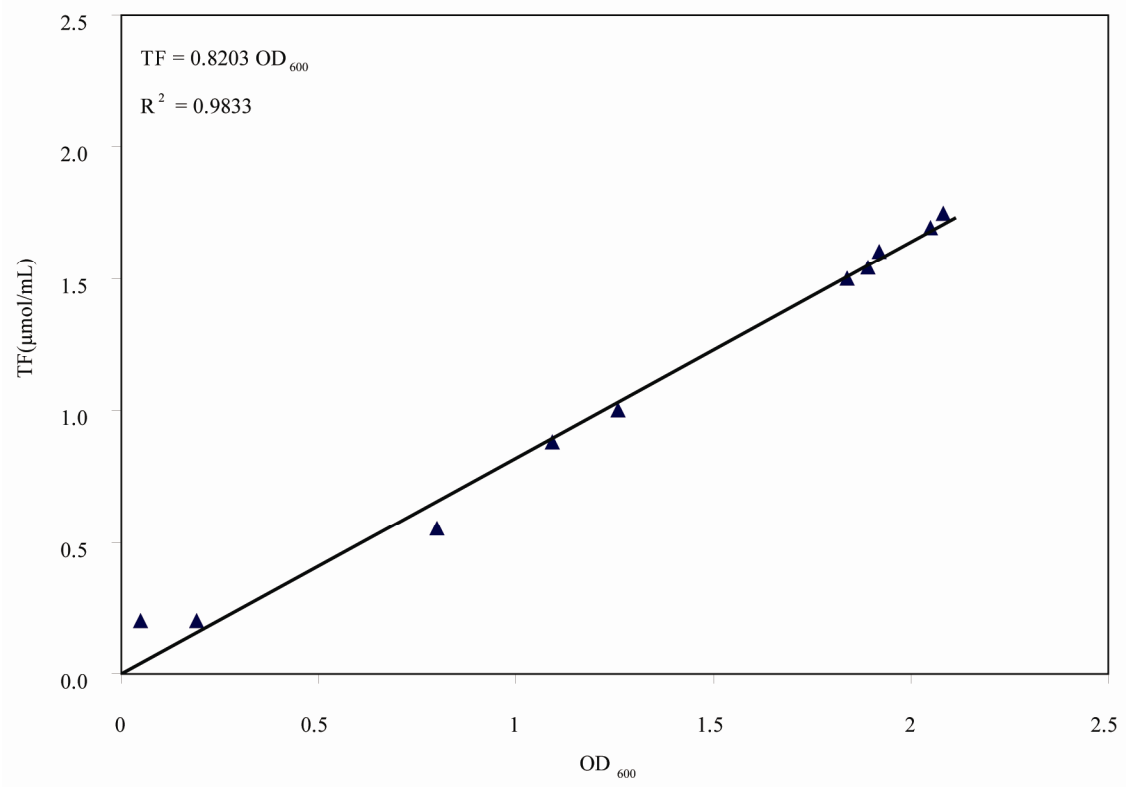


Figure 7. The relationships between OD_{600} and TF.

time do not appear to be significant and 1 hour incubation time seems to be more practical as it produced a measurable amount of TF.

There have been reports in the literature of higher TF yields as a result of increasing incubation temperatures for mixed microbial populations in soil [31] and for the fungus *A. niger* in chitin [27]. Mersi and Sehinne [23] reported that increases in incubation temperature from 25˚C to 40˚C significantly increased the dehydrogenase activity but further increases in incubation temperature to 50˚C resulted in a rapid decrease of Iodonitrotetrazolium Chloride (INT) reduction. Tiquia *et al*. [32] reported the highest level of dehydrogenase activity in yard trimming composting at temperatures in the range of 30˚C - 35˚C. Xie *et al*. [8] reported that the maximum TF yield of algae (*Chlorella* algae) in fresh water was reached at a temperature of 32˚C. Zhao *et al*. [33] reported higher (107 folds) dehydrogenase activity for heavy metal-organic contaminated sewage river sediment at incubation temperature of 30˚C than that of 4˚C. In this study, the lower TF yields observed at 40˚C and 50˚C may be due to the inhibition of the enzymatic activity of *S. venezuelae*. *S. venezuelae* are soil bacteria and, therefore, achieve optimal growth and activity within the temperature range of 22˚C - 30˚C [34]. Doull *et al*. [35] reported decreasing growth for *S. venezuelae* at temperatures higher than 37˚C compared to that observed at a temperature of 27˚C. Based on the results obtained in this study, an incubation temperature within the range of 22˚C - 30˚C is the preferred temperature for conducting the dehydrogenase test with TTC for *S. venezuelae*

5. CONCLUSIONS

The dehydrogenase activity test using triphenyl tetrazolium chloride (TTC) was employed to measure the growth and activity of *Streptomyces venezuelae* in MYM broth. The results showed high correlation between the absorbance and the TF yield. The effects of test parameters (incubation temperature, incubation time and medium pH) were evaluated in order to determine the optimum test conditions. The three parameters significantly affected the TF yield. There also seem to be significant interactions between the medium pH, incubation temperature and incubation time. In general, a pH of 6 gave the high TF yields and a pH of 9 gave higher TF yield. Lower TF yields were observed at 40˚C and 50˚C and higher yields occurred at 22˚C and 30˚C The increases in TF yield due to increase in incubation time were not significant. The recommended conditions for the reduction of TTC and the extraction of high amount of TF are 1 hour incubation at a temperature of 30˚C and a me-

dium pH of 6 followed by three extractions of TF with methanol.

6. ACKNOWLEDGEMENTS

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